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# ATP AS A SYMPATHETIC NEUROTRANSMITTER

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# CONTENTS

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# PUBLICATIONS

1). Shatarat, A., Dunn, W.R. & Ralevic, V. (2009). Sympathetic cotransmission of noradrenaline and adenosine triphosphate (ATP) in porcine mesenteric arteries. Winter Meeting, London, UK, British Pharmacological Society, pa2online.org/abstracts/Vol7Issue4abst031P.pdf

2). Shatarat, A., Dunn, W.R. & Ralevic, V. (2010). Pre-constriction increases nerve-mediated responses in rat pressurised mesenteric arteries. Winter Meeting, London, UK, British Pharmacological Society, pa2online.org/abstracts/Vol8Issue1abst002P.pdf

3). Shatarat, A., Dunn, W.R. & Ralevic, V. (2010). ATP acts as a functional sympathetic neurotransmitter in the porcine perfused mesentery after raising tone. 2nd UK Purine Symposium, Nottingham, UK, Purinergic Signal. **7**(1), 158

# ABSTRACT

ATP has been shown to be a sympathetic neurotransmitter in blood vessels. However, its relative importance has been shown to be influenced by the experimental conditions employed such as alteration of the vascular tone. Thus the main aim was to raise the tone of vascular preparations and to further examine sympathetic neurotransmission in these preparations. Porcine whole mesenteries were perfused with physiological buffer and changes in pressure recorded or different sized mesenteric arteries were isolated and set up for isometric recording.

Responses to electrical field stimulation (EFS) were obtained under basal and raised tone conditions induced by U46619, a thromboxane $A_2$ mimetic. The nature of the neurotransmitters involved in the mediation of the electrically-evoked responses was assessed using an $\alpha_1$-adrenoceptor antagonist, prazosin and/or the P2X receptor desensitizing agent, α,β-methyleneATP, an $\alpha_2$-adrenoceptor RX811059 antagonist, and a neuropeptide Y $Y_1$ receptor antagonist BIBP3226. In separate experiments, responses to nerve stimulation were investigated in rat mesenteric small arteries pressurized to 90 mmHg. The effects of a selective $\alpha_1$-adrenoceptor antagonist, YM-12617, and selective $P2X_1$ receptor antagonist, NF-449, on the electrically-evoked response were determined.

Under basal tone conditions the electrically-evoked contractile responses in porcine whole mesenteric bed and isolated arteries were exclusively mediated by noradrenaline (NA) since they were inhibited by prazosin. However, under conditions of raised tone, the electrically-evoked responses were enhanced and a role for ATP was evident since these responses were sensitive to α,β-methyleneATP. Responses to exogenous NA and α,β-methyleneATP were also enhanced at raised tone indicating a postjunctional mechanism of enhancement. Nifedipine attenuated the enhanced responses to EFS and α,β-methyleneATP suggesting a possible role for L-type calcium channels in the mediation of the enhanced responses. In rat pressurised mesenteric arteries the electrically-evoked vasocontractile responses were sensitive to YM-12617 and NF-449, indicating that NA and ATP were involved in the mediation of these responses. Raising tone with U46619 in these arteries enhanced the electrically-evoked contractile response; under these conditions responses were sensitive to both YM-12617 and NF-449.

The present study supports the observation that ATP becomes a more important sympathetic neurotransmitter under conditions of raised tone in contrast to when tone is absent. In porcine mesenteric vascular preparations NA predominates as the main sympathetic neurotransmitter under conditions of basal tone. However, when tone was raised the responses were enhanced and a role for ATP became evident.

# ABBREVIATION

| | |
|---|---|
| **α,β-methyleneATP** | α,β-methyleneATP |
| **Ach** | Acetylcholine |
| **ADP** | Adenosine diphosphate |
| **ANOVA** | Analysis of variance |
| **ATP** | Adenosine 5-triphosphate |
| **CGRP** | Calcitonin gene-related peptide |
| **EDHF** | Endothelium-derived hyperpolarizing factor |
| **EFS** | Electrical field stimulation |
| **EJC** | Excitatory junction current |
| **EJP** | Excitatory junction potential |
| **PGP 9.5** | General neuronal marker protein gene product 9.5 |
| **ET-1** | Endothelin-1 |
| **GPCRs** | G-protein-coupled receptors |
| **IJP** | Inhibitory junction potentials |
| **FRC** | Frequency response curve |
| **NA** | Noradrenaline |
| **NANC** | Non-adrenergic, non cholinergic |
| **NKA** | Neurokinin A |
| **NO** | Nitric oxide |
| **NPY** | Neuropeptide Y |
| **MT** | Myogenic tone |
| **PBS** | Phosphate buffered saline |
| **RBC** | Red blood cells |
| **SP** | Substance P |
| **SR** | Sacroplasmic reticulum |
| **SV2** | Synaptic vesicle proteoglycan |
| **TH** | Tyrosine hydroxylase |
| **TTX** | Tetrodotoxin |

# CHEMICAL NAMES

| | |
|---|---|
| **U46619** | Thromboxane mimetic 9,11-dideoxy-11alpha,9 alpha-epoxymethanoprostaglandin F2alpha |
| **Capsaicin** | 8-methyl-N-vanillyl-6-nonenamide |
| **YM-12617** | 5-[2-[[2-(2-ethoxyphenoxy)ethyljaminojpropyl]-2-methoxybenzene-sulfonainide HC1 |
| **NF-449** | 4,4',4",4"'(Carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakis-1,3-benzenedisulfonicacidoctasodium salt |

# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 ROLE OF BLOOD VESSELS IN THE REGULATION OF SYSTEMIC BLOOD PRESSURE

Systemic blood pressure is the product of the cardiac output and systemic peripheral vascular resistance. The homeostatic systems that influence blood pressure are neural regulation, arterial baroreceptors and chemoreceptors, regulation of fluid volume, and humoral regulation (Guyton, 2005). Apart from the regulation of fluid volume, which is mainly controlled by the action of the kidneys, other factors that regulate systemic blood pressure mainly target blood vessels, with small arteries being crucial in the control of peripheral resistance and hence in regulating blood pressure. Blood vessels diameter is controlled by the three layers that compose the blood vessels. The innermost layer of blood vessels, which is called the endothelium, can actively contribute to the contractile status of blood vessels by releasing several biologically active substances including nitric oxide (NO) (Furchgott et al., 1984), prostacyclin (Moncada et al., 1979), as well as endothelium derived hyperpolarizing factor (EDHF) (Taylor and Weston, 1988). The outermost layer of blood vessels, called the adventitia, contains perivascular nerves which are usually of two types; sympathetic and sensory (also called sensory-motor or capsaicin-sensitive sensory nerves). Both mediate their functions by releasing different neurotransmitters. Between the endothelial and adventitial layers is a layer of smooth muscle cells which responds to the different signals released from endothelium and perivascular nerves in the adventitia to enable

the blood vessel to alter its diameter. Thus the function of blood vessels is under a dual regulation of endothelium and perivascular nerves (Burnstock, 1990). Furthermore, blood vessels are also regulated by hormones within the blood and formed elements of blood such as red blood cells (RBC). RBC act as a sensor for hypoxia thus when $O_2$ levels become low RBC release adenosine triphosphate (ATP) which stimulates vasodilatation (Dietrich et al., 2000). Therefore, blood vessel contractility is orchestrated by endothelium, blood borne factors and perivascular nerves.

However, another mechanism which has been shown to be involved in the regulation of blood flow is the ability of small arteries, especially arterioles, to develop myogenic tone (MT) (Johnson, 1981). MT is the ability of small blood vessels to constrict in response to increases in intraluminal pressure or to relax in response to decreases in blood pressure regardless of the neuronal, hormonal and metabolic influences (Davis and Hill, 1999).

## 1.2 PERIVASCULAR NERVES

### 1.2.1 SENSORY NERVES

Blood vessels are innervated by sensory nerves in addition to sympathetic nerves. These nerves are called sensory-motor or capsaicin-sensitive sensory nerves due to their sensitivity to capsaicin, the active ingredient of chilli pepper (Holzer, 1991a). The common conception about sensory nerves in general is that they convey information from the periphery to the central nervous system. Interestingly in some organs including blood vessels it has been demonstrated

that sensory-motor nerves act as effector neurons by releasing neuropeptides which act as neurotransmitters to alter vascular diameter and hence control blood flow (Maggi and Meli, 1988). The peptides include calcitonin gene-related peptide (CGRP), substance P and neurokinin A (NKA). Their release has been demonstrated in several blood vessels. For example, stimulation of the perivascular nerves of rat mesenteric arteries causes the release of CGRP which causes vasorelaxation (Kawasaki et al., 1988). The function of sensory-motor nerves can be divided into local effects, achieved by the release of neurotransmitters (Holzer, 1991b), and central effects through different reflexes, including thermoregulatory, neuroendocrine and cardiovascular, to maintain body homeostasis (Rubino and Burnstock, 1996). It has previously been shown that depletion of sensory afferent nerves of their neurotransmitters using capsaicin, enhanced the response to electrical field stimulation (EFS) in the rat isolated mesenteric vascular bed (Li and Duckles, 1992). Similar observation has also been reported recently in the rat isolated mesenteric vascular bed (Pakdeechote et al., 2007), most likely as a result of allowing sympathetic nerves to mediate the responses without functional antagonism from sensory nerves (Ralevic and Kendall, 2002).

### 1.2.2 Sympathetic nerves

Each sympathetic nerve stores catecholamines in its terminal vesicles, and since catecholamines react with formaldehyde to become highly fluorescent, the identification of catecholamines in sympathetic nerves in different organs and tissues became possible (Falck, 1962). The presence and distribution of sympathetic nerves has been demonstrated in different blood vessels in many

species, for example, in terminal arterioles of the rat mesentery (Furness, 1973), pulmonary arteries of the monkey (El-Bermani, 1978), and the superior mesenteric artery and vein of rat (Nilsson et al., 1986). Furthermore, the retrograde fluorescent dye, fast blue, has also been used to identify sympathetic neurons. For example, fast blue revealed sympathetic postganglionic neurons supplying mesenteric arteries of the rat (Sheppard, 1986). Recently the presence of dense sympathetic innervation in human mesenteric arteries and veins has been demonstrated using immunohistochemical staining coupled with electron microscopy (Birch et al., 2008).

## 1.3 SYMPATHETIC COTRANSMISSION

Postganglionic sympathetic nerve activation produces biological effects via the release of neurotransmitter. Initially, noradrenaline (NA) was considered to be the sole neurotransmitter released from sympathetic neurons until the concept was challenged by Burnstock who suggested the possible release of more than one neurotransmitter from the same neuron (Burnstock, 1976). Since then, many studies have shown that neurons can release more than one neurotransmitter. For example, adenosine triphosphate (ATP) was proposed by Burnstock as a sympathetic neurotransmitter (Burnstock, 1972). Thereafter, it was shown that neuropeptide Y (NPY) was also released from sympathetic nerves (Lundberg et al., 1983). These discoveries led to the development of the concept of cotransmission.

Cotransmission is now a well established concept in the central and peripheral nervous systems. However, many questions remain about the physiological

significance of cotransmission. It has been suggested that cotransmission allows a synergistic relationship between different neurotransmitters, promotes prejunctional neuromodulation and allows differential release of cotransmitter during different firing patterns of nerves. An example of a synergistic relationship between NA as main transmitter and ATP as a cotransmitter in the smooth muscle of rat mesenteric arteries and vas deferens has been shown, and it has been suggested that ATP can cooperate with NA to cause smooth muscle contraction in a synergistic manner (Ralevic and Burnstock, 1990, Ventura et al., 2003). In addition, it has been suggested that one neurotransmitter can lead to the sequestration of $Ca^{2+}$ and thus facilitate the neurotransmission of the other neurotransmitter. For example, it has been demonstrated in the mouse vas deferens that upon EFS, neuroeffector $Ca^{2+}$ transients (NCTs) were obtained; these NCTs were abolished by the P2X receptor agonist and desensitising agent, α,β-methyleneATP indicating that NCTs were mediated by ATP through P2X receptors (Brain et al., 2002b). Later Brain and co-workers showed that NCTs led to sequestration of $Ca^{2+}$ in intracellular stores for subsequent $Ca^{2+}$ release and they suggested that this may provide a mechanism for purinergic enhancement of noradrenergic neurotransmission (Brain et al., 2003).

It has also been shown that cotransmission promotes more complex patterns of presynaptic neuromodulation where the cotransmitter can play an important role in the regulation of the release of the main transmitter. NPY for example inhibits the release of NA and ATP in the guinea-pig vas deferens (Ellis and Burnstock, 1990) or enhances the release of NA in rabbit blood vessels

(Edvinsson et al., 1984), even though it has very little direct postjunctional effects of its own. Recent studies showed that cotransmission can be involved in postjunctional neuromodulation as well. For example, in mouse urinary bladder smooth muscle, ATP which is co-released with acetylcholine (Ach) suppressed the subsequent muscarinic-mediated increases in excitability and force generation via the activation of postsynaptic P2X receptors (Heppner et al., 2009).

Furthermore, it has been shown that NA release is favoured at higher frequencies while NPY and ATP release is favoured at lower frequencies of EFS in rat tail artery (Bradley et al., 2003), which means that in response to different parameters of stimulation the presence of more than neurotransmitter would allow different responses of nerves to take place.

### 1.3.1 Noradrenaline

It was originally believed that sympathetic nerves released adrenaline. This proposal arose from results obtained in experiments using the perfused rabbit ear and frog heart in which the active substance released by adrenergic nerves produced similar responses to exogenous adrenaline (Gaddum and Kwiatkowski, 1939). However, analysis of homogenised splenic nerves of the ox showed the presence of NA in submicroscopic structures of adrenergic axons (Hillarp, 1956). In addition, biochemical studies showed the presence of enzymes responsible for noradrenaline synthesis in chromaffin cells and neurons, and that both tissues were capable of storing and releasing catecholamines (Blaschko, 1957). Further experiments demonstrated that NA

was formed from tyrosine in homogenates of sympathetic nerve tissue, indicating that tyrosine acts as the main precursor of NA synthesis (Goodall and Kirshner, 1958). Tyrosine is transported by a noradrenaline linked carrier into the exoplasm of adrenergic neurons where it is converted into dopa by tyrosine-3-monoxygenase. Thereafter, L -amino acid decarboxylase converts dopa to dopamine, and this is transported into sympathetic vesicles where it undergoes hydroxylation to form noradrenaline (Blaschko, 1957). Thus, the first two steps of NA formation take place outside the storage vesicles while the last step occurs inside (Stjarne and Lishajko, 1967). Further studies, using cell fractionation, fluorescence and electronmicroscopy methods revealed the presence of small and large dense-cored vesicles containing NA in sympathetic nerve terminals of rat vas deferens (Bisby and Fillenz, 1970).

The release of NA from small and large dense-cored vesicles is a $Ca^{2+}$ dependent process following invasion of the nerve varicosity by an action potential. It is noteworthy that large vesicles are involved in the release of noradrenaline at higher frequencies while the small vesicles are involved at lower frequencies (Stjarne, 1989). Direct evidence for the release of NA was initially shown using the measurement of tritium released from tissues preloaded with $^{3}$[H] NA in rabbit main pulmonary artery (Su and Bevan, 1970). Furthermore, the release of endogenous NA from sympathetic nerves has been measured using high performance liquid chromatography in rat tail artery (Buchholz and Duckles, 1992), as well as in the rat mesenteric artery (Ralevic and Kendall, 2002). The introduction of continuous amperometry, which measures the increase in NA concentration at the adventitial surface of

the vessel as an oxidation current using carbon fibre electrode (Gonon et al., 1993), has led to monitoring of the real time of NA release on an impulse-by-impulse basis in rat tail artery during trains of stimulation (Brock et al., 1997). More recent studies in which an amperometric method was used showed that the release of NA is regulated by activation of $\alpha_2$-adrenoceptors and suggested that the released NA is cleared by diffusion and uptake in sympathetic nerves supplying rat mesenteric arteries (Dunn et al., 1999). Moreover, the spontaneous packeted release of NA, most likely from large dense-cored vesicles has been demonstrated from sympathetic nerve terminals in rat mesenteric arteries in vitro (Brock et al., 2000).

However most of the evidence which indicates that noradrenaline is the main sympathetic neurotransmitter has been obtained from pharmacological studies. For example, responses to sympathetic nerve stimulation were abolished by the $\alpha_1$-adrenoceptor antagonist prazosin, providing evidence that NA was the main neurotransmitter in guinea-pig, rat and rabbit small blood vessels (Angus et al., 1988).

#### 1.3.1.1 Adrenoceptors

In 1906 Dale, noticed some responses to adrenoceptor stimulation which were not blocked by classical adrenoceptor antagonists such as ergot alkaloids suggesting more than one adrenoceptor existed (Dale, 1906). However it was not until 1948 when Ahlquist used different sympathomimetic agonists, that the existence of two adrenoceptor subtypes was proposed; one for excitatory events, which he called α-adrenoceptors and another for inhibitory events,

which he called β-adrenoceptors (Ahlquist, 1948). The discovery of dichloroisoprenaline, which was the first agent capable of antagonising β-adrenoceptor-mediated responses selectively, confirmed the existence of α- and β- subtypes (Powell and Slater, 1958).

#### 1.3.1.2 *β*-adrenoceptors

Because of the greater availability of agonists and antagonists acting at β-adrenoceptors many studies initially focused on β-adrenoceptors. On the basis of different potency to agonists, Lands and co-workers concluded that there were two subtypes of β-adrenoceptors; the $\beta_1$-adrenoceptor (with equal sensitivity to both NA and adrenaline and dominance in cardiac and gastrointestinal tissues) and the $\beta_2$-adrenoceptor (with less sensitivity to NA and greater presence in vascular, uterine and airway smooth muscles) (Lands et al., 1967).

As more experiments were performed examining β-adrenoceptors, a number of tissues were identified which displayed β-adrenoceptor-mediated responses but which were relatively unresponsive to $\beta_1$- and $\beta_2$- agonists. This raised the possibility of the existence of a third β-adrenoceptor subtype. The development of selective β-adrenoceptor agonists such as BRL 28410 and BRL 37344 has shown atypical β-adrenoceptor expression in some tissues (Arch et al., 1984, McLaughlin and MacDonald, 1990). Furthermore, responses to different beta-adrenergic agonists were studied on adipose cells of the rat using betaxolol ($\beta_1$-adrenoceptor selective antagonist) or ICI 118551 ($\beta_2$-adrenoceptor selective antagonist). In this study it has been shown that regulation of lipolysis in

adipose tissue was not mediated exclusively by either $\beta_1$- or $\beta_2$-adrenoceptors as it was originally assumed. Therefore they proposed a third β-adrenoceptor termed the $\beta_3$-adrenoceptor (Tan and Curtis-Prior, 1983).

There was speculation about the presence of a fourth subtype of β-adrenoceptor mediating relaxation in rat aorta using the $\beta_3$-adrenoceptor partial agonist CGP-12177 (Brawley et al., 2000). However it was demonstrated later that the $\beta_4$-adrenoceptor does not exist in mice, and it was concluded that the effects of CGP-12177 were due to an atypical interaction of this compound with $\beta_1$-adrenoceptors (Kaumann et al., 2001).

The use of cloning confirmed the presence of the human $\beta_1$-adrenoceptor (Frielle et al., 1987), mammalian $\beta_2$-adrenoceptor (Dixon et al., 1986) and human $\beta_3$-adrenoceptor (Emorine et al., 1989). Thus, to the best of our knowledge β-adrenoceptors are of three types; $\beta_1$-adrenoceptors responsible for the regulation of contractility and heart rate, $\beta_2$-adrenoceptors mediating vasodilatation evoked by sympathomimetic agonists and $\beta_3$-adrenoceptors which are predominant in adipose tissue and regulate biolysis (Guimaraes and Moura, 2001, Alexander, 2009).

#### 1.3.1.3 *α*-adrenoceptors

In 1957, Brown and Gillespie showed that dibenamine, which is an irreversible α-adrenoceptor antagonist, increased the output of NA in venous blood from the cat spleen following nerve stimulation and they suggested that this effect was due to blockade of postjunctional α-adrenoceptors. Their reasoning was that the released NA could not combine with α-adrenoceptors on the effector

cells leading to an increase of NA output (Brown and Gillespie, 1957). However their conclusions were not consistent with the results of other experiments where phenoxybenzamine, which is an irreversible nonspecific α-adrenoceptor blocker, failed to increase the NA output when the reuptake of NA had been prevented by cocaine (NA reuptake inhibitor), which indicated that the increase of NA output was due to the inhibition of neuronal uptake (Thoenen et al., 1964). Furthermore, investigation of the influence of unlabelled NA on the release of previously stored $^{14}$C-NA in the presence of cocaine and phenoxybenzamine in the isolated perfused rabbit heart revealed that the extracellular NA inhibited the release of intraneuronal NA evoked by nerve stimulation, and this inhibition was mediated by α-adrenoceptors localised on the neuronal membrane. In other words it was shown for the first time that NA can inhibit its own release by a feedback mechanism (Starke, 1972). The availability of certain adrenergic agonists such as clonidine, and antagonists such as phenoxybenzamine, helped in discriminating between pre- and postjunctional α-adrenoceptors which led Langer to propose an anatomical basis for subdivision of α-adrenoceptors into the postjunctional α-adrenoceptor, which mediates effector organ responses, and the prejunctional α-adrenoceptor, which regulates neurotransmitter release (Langer, 1974). Later, Berthelsen and Pettinger proposed a functional basis for subdivision of α-adrenoceptors. Accordingly receptors mediating excitation have been termed $\alpha_1$-adrenoceptors while those mediating inhibition were termed $\alpha_2$-adrenoceptors (Berthelsen and Pettinger, 1977).

However, the development of the highly selective $\alpha_1$-adrenoceptor antagonist prazosin and $\alpha_2$-adrenoceptor antagonist yohimbine enabled Drew and Whiting, to demonstrate that the vasoconstrictor responses in rat and cat blood vessels were inhibited by prazosin as well as yohimbine, an indication that the vasoconstrictor responses were mediated not only by $\alpha_1$ but also by $\alpha_2$ adrenoceptors (Drew and Whiting, 1979). Thus neither the anatomical nor the functional classification fulfils the truth of α-adrenoceptors. This led Starke to propose a new classification based on pharmacological potencies of different adrenoceptor agonists and antagonists. Thus $\alpha_1$-adrenoceptors are activated by phenylephrine, methoxamine and blocked by prazosin while $\alpha_2$-adrenoceptors are activated by UK-14,304 or BHT933 and blocked by rauwolscine or idozoxan (Starke, 1981).

The use of cloning methods have shown three subtypes of $\alpha_1$-adrenoceptors; $\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$ (Hieble et al., 1995) and three subtypes of $\alpha_2$-adrenoceptors; $\alpha_{2A}$, $\alpha_{2B}$, and $\alpha_{2C}$ (Kobilka et al., 1987, Regan et al., 1988, Lomasney et al., 1990). Thus, based on pharmacological potencies of different adrenoceptor agonists and antagonists in functional studies and cloning as well as radioligand binding data, at present there are three subtypes of $\alpha_1$- and $\alpha_2$-adrenoceptors. $\alpha_1$-adrenoceptor subtypes are $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$, while $\alpha_2$-adrenoceptor subtypes are $\alpha_{2A}$, $\alpha_{2B,}$ and $\alpha_{2C}$ (Alexander, 2009).

The relative importance of the different subtypes of $\alpha_1$-adrenoceptors in regulation of blood vessel contractility varies between species and vascular beds (Guimaraes and Moura, 2001). In addition, activation of different subtypes of $\alpha_1$-adrenoceptors either by circulating NA or by neurally released

NA is another factor that may influence the involvement of different $\alpha_1$-adrenoceptors subtypes in the regulation of the of blood vessels contractility. For example, NA released from sympathetic nerves in response to EFS, activated $\alpha_{1B}$- and in part $\alpha_{1D}$-adrenoceptors, whereas exogenous NA activated $\alpha_{1A}$-adrenoceptor in canine splenic arteries (Yang and Chiba, 2001). Furthermore, it has been shown that $\alpha_{1A}$-adrenoceptors mediated contraction in response to both exogenous and neurally released NA while $\alpha_{1D}$-adrenoceptors were not involved in responses to exogenous NA in rat femoral resistance arteries (Zacharia et al., 2004). In contrast, it has been shown that $\alpha_{1A}$-adrenoceptors mediated responses to both exogenous and neurally released NA in the isolated perfused mesentery of rat (Williams and Clarke, 1995).

#### 1.3.1.3.1 *α*-adrenoceptors function

$\alpha_1$-adrenoceptors are mainly coupled to $G_{q/11}$-protein. Their activation stimulates phospholipase C enzyme which promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate producing inositol triphosphate ($IP_3$) and diacyl glycerol (DAG). $IP_3$ and DAG act as second messengers mediating intracellular $Ca^{2+}$ release from intracellular pools (Zhong and Minneman, 1999). $\alpha_2$-adrenoceptors are in general negatively coupled to adenylyl cyclase through a $G_i$-protein (Bylund et al., 1994, Wise et al., 1997), their activation causes a reduction in intracellular adenosine 3' : 5'-cyclic mono-phosphate (cyclic AMP) production (Roberts et al., 1998).

In regard to vasculature, postsynaptic $\alpha_1$-adrenoceptors have been primarily shown to mediate contractile responses to sympathetic nerve activation, as shown in different blood vessels in different species. For example, electrically-

evoked contractile responses were sensitive to $\alpha_1$-adrenoceptors antagonism in rabbit hindlimb (Madjar et al., 1980), isolated pulmonary artery of the rabbit (MacLean et al., 1993), horse penile resistance arteries (Simonsen et al., 1997) and guinea-pig mesenteric arteries (Smyth et al., 2000). However, a role for $\alpha_2$-adrenoceptors in mediation of postjunctional responses to the $\alpha_2$-adrenoceptor agonist UK 14304 has been demonstrated in small resistant vessels, for example, in human subcutaneous resistance vessels (Nielsen et al., 1989). In large conducting arteries on the other hand activation of $\alpha_2$-adrenoceptors needed experimental manipulation. For example, increasing of perfusion pressure by arginine vasopressin in the isolated vascular bed of the rat tail (Templeton et al., 1989), or the presence of an unrelated vasoconstrictor such as angiotensin II in rabbit isolated distal saphenous artery (Dunn et al., 1989) and the presence of U46619 and forskolin in porcine isolated ear artery (Roberts et al., 1998). The involvement of postjunctional $\alpha_2$-adrenoceptors in the mediation of neurogenic contractile responses to sympathetic nerve activation has also been shown to require experimental manipulation. For example, in the presence of angiotensin II the $\alpha_2$-adrenoceptors antagonist rauwolscine inhibited the electrically-evoked responses in the rabbit isolated distal saphenous artery (Dunn et al., 1991b). However, a role for $\alpha_2$-adrenoceptors in the mediation of the electrically-evoked vasoconstriction without experimental manipulation has been shown in the rat tail artery, although their involvement was frequency- and train length-dependent (Bao et al., 1993).

In regard to human blood vessels, in vivo experiments demonstrated the presence of postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptors by measuring the influence on forearm blood flow induced by intra-arterial infusions of selective $\alpha_1$- and $\alpha_2$-adrenoceptor agonists and antagonists (Jie et al., 1984). In addition, it has been shown that presynaptic $\alpha_2$-adrenoceptors modulate NA release from sympathetic nerve endings via a negative feedback mechanism in humans (Jie et al., 1987). More recent studies showed the involvement of both postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptors in the mediation of the electrically-evoked vasoconstrictor responses in human gastroepiploic artery (Fukui et al., 2005).

### 1.3.2 Adenosine triphosphate (ATP)

#### 1.3.2.1 ATP structure

ATP is made of a nitrogenous base (adenine), a sugar (ribose) and a chain of three ionised groups (phosphates) bound to the ribose (see Fig. 1.1). However, each group is made by an independent metabolic route. In neurons ATP is produced by oxidative phosophorylation of glucose which is usually comes from the extracellular fluid (Sperlágh and Vizi, 1996). ATP was first identified in muscle extracts in 1929 (Fiske and Subbarow, 1929). However, it was following identification of its role in the breakdown of glucose to lactic acid that marked its role in cellular energy (Lipmann, 1941), and since then ATP has been recognised as the source of cellular energy. The recognition of this important role led to the belief that ATP could not be an extracellular

transmitter, since it was believed that cells would not sacrifice such an important biomolecule for this purpose.

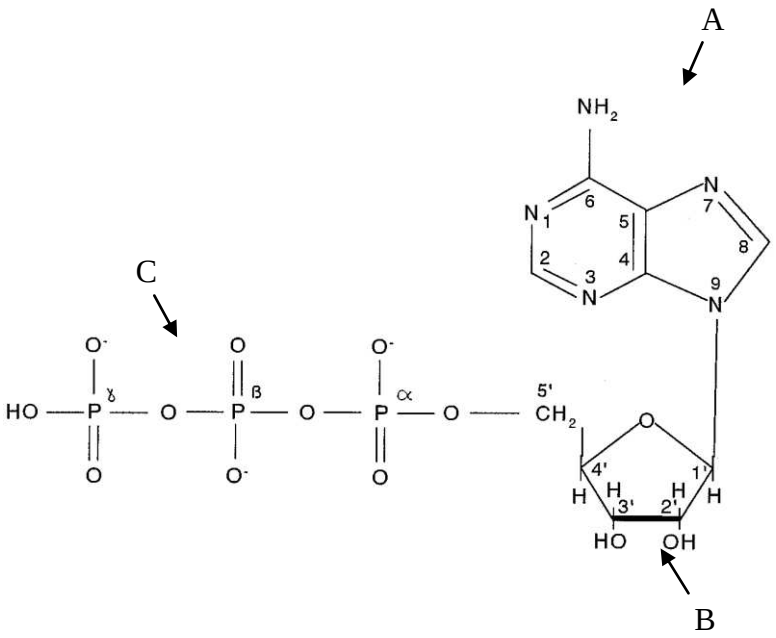



Fig. 1.1 Diagram shows that The ATP molecule is built up from three chemically different parts A) the adenine ring, B) the ribose sugar and C) the triphosphate chain.

### 1.3.2.2 ATP storage, release and degradation

The presence of ATP has been demonstrated in both large and small dense-cored sympathetic vesicles (Lagercrantz, 1971). Vesicular exocytotic release seems to be the mechanism by which ATP is released (Pankratov et al., 2006, Pankratov et al., 2007). However there is a debate about the mechanisms involved in ATP transport, since ATP is a relatively large molecule that cannot pass through the membrane by simple diffusion. Thus, it has been suggested a non vesicular ATP release in some cells which may involve ATP-binding

cassette transporter, plasmalemmal voltage-dependent anion channels and connexin or pannexin hemichannels (Scemes et al., 2007). It has been recently demonstrated in ATP-secreting cells, that this transporter might be the vesicular nucleotide transporter (VNUT) which belongs to the SLC17 anion transporter family (Sawada et al., 2008).

The release of ATP upon depolarizing stimuli has been shown in different blood vessels including rabbit pulmonary artery and rat caudal artery (Westfall et al., 1987, Takeuchi et al., 1994). In addition, ATP release has been clearly demonstrated in cultured sympathetic nerve terminals where tetrodotoxin (neurone toxin) inhibited this (Richardson and Brown, 1987, Von Kügelgen et al., 1994). The release of ATP upon nerve stimulation has also been shown to be a $Ca^{2+}$-dependent process (Brock and Cunnane, 1999). Using the luciferin-luciferase assay Kirkpatrick and Burnstock showed the release of ATP from sympathetic nerves in the guinea-pig vas deferens (Kirkpatrick and Burnstock, 1987). An indirect measurement of ATP released from sympathetic nerve endings in rat mesenteric arteries was obtained through the recording of the excitatory junction potentials (EJPs) from smooth muscles (Brock et al., 2000). However, more direct evidence for the release of ATP from the varicosities of sympathetic nerves comes from the use of the confocal $Ca^{2+}$ imaging technique where it has been demonstrated that the intermittent release of ATP from nerve terminals elicited focal smooth muscle $Ca^{2+}$ transients in the mouse vas deferens (Brain et al., 2002a).

Once ATP is released into the extracellular fluid, it is regulated by cell surface-located enzymes named ectonucleotidases. Ectonucleotidases are capable of

hydrolyzing ATP to ADP, AMP and adenosine (Gordon, 1986, Zimmermann, 2006). However, it has been shown that stimulation of sympathetic nerves innervating the guinea-pig vas deferens releases not only neuronal ATP, but also soluble nucleotidases which led to the breakdown of ATP to adenosine (Kennedy et al., 1997, Todorov et al., 1997)

#### 1.3.2.3 Recognition of ATP as an extracellular neurotransmitter

In 1960 Burnstock and co-workers obtained inhibitory junction potentials (IJPs), hyperpolarisations of smooth muscle produced by stimulation of inhibitory nerves, from intestinal smooth muscle as a result of the stimulation of noradrenergic noncholinergic (NANC) nerves. The nature of the transmitter which was involved in the transmission of the IJPS remained unclear (Burnstock and Prosser, 1960). It was not until a decade later that Burnstock and co-workers uncovered the nature of the transmitter which was responsible for the transmission of IJPs. They investigated the vagal nonadrenergic inhibitory responses of guinea-pig and toad stomachs where they showed the presence of ATP in the vascular perfusates. Thus, they concluded that ATP was the transmitter substance released by nonadrenergic inhibitory nerves (Burnstock et al., 1970). Furthermore, it was demonstrated that ATP has postjunctional effects on the smooth muscle of vas deferens of guinea-pig (Westfall et al., 1978). Subsequent studies showed that neurogenic responses in the vas deferens of the guinea pig were blocked by an ATP receptor antagonist arylazido aminopropanol ATP (ANAPP3) (Fedan et al., 1981). Furthermore, the ATP receptor agonist and desensitizing agent α,β-methyleneATP abolished the EJPs obtained to EFS in guinea-pig vas deferens

(Sneddon and Burnstock, 1984). However, in blood vessels, it was demonstrated that exogenous ATP evoked vasoconstriction followed by vasorelaxation in isolated rabbit portal vein (Kennedy and Burnstock, 1985). Electrical nerve stimulation produced EJPs that were insensitive to both prazosin and yohimbine, while abolished by α,β-methyleneATP suggesting that ATP was the sole transmitter in rabbit jejunal arteries (Ramme et al., 1987). Furthermore, α,β-methyleneATP blocked the neurogenic response in rat mesenteric artery (Angus et al., 1988).

Meanwhile the appearance of cotransmission as a new concept in autonomic transmission opened the horizon to further investigate the possibility that ATP might be a cotransmitter with noradrenaline in sympathetic neurones, although the presence of ATP and catecholamines in specific granules of adrenal medulla had been shown as early as 1956 (Carlsson and Hillarp, 1956).

#### 1.3.2.4 COTRANSMISSION OF ATP AND NORADRENALINE

The presence of ATP together with NA in both small and large dense-cored sympathetic vesicles was demonstrated by Lagercrantz (Lagercrantz, 1971). Tritium labelled adenosine and NA were used to investigate the sympathetic nerves supplying the rabbit portal vein where it was found that ATP was released together with NA (Su, 1975). The presence of ATP as a cotransmitter with NA was shown in isolated mesenteric artery of the dog where EFS of the perivascular nerves produced responses that were not completely blocked by the $\alpha_1$-adrenoceptor blocker prazosin or by the $\alpha_2$-adrenoceptor blocker yohimbine, but α,β-methyleneATP blocked the prazosin resistant component,

which gave a strong indication that part of the responses was mediated by ATP (Muramatsu, 1986). Furthermore, it has been shown that ATP is a cotransmitter with NA in sympathetic nerves of the rabbit hepatic artery (Brizzolara and Burnstock, 1990) and in guinea-pig mesenteric veins (Smyth et al., 2000).

The co-existence of ATP and NA in both small and large dense-cored sympathetic vesicles has been shown as discussed earlier. However, there have been questions to whether NA and ATP were separately released from the same subpopulations of sympathetic vesicles or from different subpopulations of sympathetic vesicles containing different proportions of NA and ATP. The answer to these questions was difficult as there was no unified method to measure the simultaneous release of both neurotransmitters. Since NA is electrochemically oxidizable (Dunn et al., 1999), whereas ATP is not, continuous amperometry has been used successfully in measuring the release of NA as it is not sensitive for ATP. Thus, researchers used continuous amperometry to measure NA release in combination with recording of the intracellular EJPs evoked by ATP as indirect method to measure ATP release (Brock and Cunnane, 1993). Results of these experiments showed that the release of ATP and NA from sympathetic nerves can be differentially modulated by drugs. For example, the $\alpha_2$-adrenoceptor antagonist, idazoxan had different effects on the oxidation currents produced by NA and EJPs evoked by the release of ATP in sympathetic nerves supplying rat mesenteric arteries (Dunn et al., 1999).

The use of α-latrotoxin (which is a peptide that evokes the exocytotic release of neurotransmitters from a variety of nerve terminals including sympathetic neurones) has shown that the release of NA is most probably from large dense-cored vesicles; conversely the release of ATP was shown to be from a different store, most likely the small dense-cored vesicles in rat mesenteric arteries (Brock et al., 2000). The results of these experiments support the differential release of NA and ATP. However, using the extracellular recording of excitatory junction current (EJC) and the fractional increase in overflow of tritium to monitor the per pulse secretion of ATP and [$^3$H] NA from sympathetic nerves in guinea-pig vas deferens, Stjärne demonstrated the presence of two classes of small vesicles (SVs). The SVs store and release either big or small ATP and NA quanta. Furthermore, he suggested that different affinity to $Ca^{2+}$ in the SVs membranes would enable the nerves to selectively secrete big quanta at low frequency and small quanta during trains at high frequency (Stjarne, 2001).

New techniques provided direct evidence for cotransmission and co-release of ATP and NA. For example, using $Ca^{2+}$ confocal imaging in vascular smooth muscle of rat mesenteric arteries, a novel type of $Ca^{2+}$ transient (termed a junctional $Ca^{2+}$ transient) occurring early in response to EFS that was followed by $Ca^{2+}$ waves developing later, has been demonstrated (Wier et al., 2009). Wier and co-workers suggested that the early junctional $Ca^{2+}$ transients were mediated by ATP and the later $Ca^{2+}$ waves were mediated by NA. Moreover, they suggested that during an early train of nerve stimulation, smooth muscle contraction is activated mainly by neurally released ATP from sympathetic

varicosities, mainly small vesicles that contain a relatively high concentration of ATP and using $Ca^{2+}$ that has entered via P2X receptors. Later during a train of nerve stimulation, NA binds to $\alpha_1$-adrenoceptors leading to the activation of second messenger resulting in the release of $Ca^{2+}$ from sacroplasmic reticulum (SR). At this time, sympathetic varicosities may release small synaptic vesicles that contain a relatively high concentration of NA (Lamont et al., 2003, Wier et al., 2009).

In summary, the detection of co-release of ATP and NA in sympathetic nerves using amperometric and neurotransmitter overflow methods has shown a differential release of ATP and NA, while the use of $Ca^{2+}$ confocal imaging suggest the co-release of packets of these neurotransmitters during low-frequency nerve stimulation (Brain, 2009). Therefore, ATP and NA synergise resulting in a quick and sustainable contraction; ATP is generally released during short bursts of sympathetic nerve activation leading to a rapid response since P2X receptors are ionotropic channels, whereas prolonged periods of activation favour the release of the NA which acts more slowly due to G protein coupling and the involvement of second messengers.

#### 1.3.2.5 Factors affecting ATP as a sympathetic neurotransmitter

Since the recognition that ATP can act as a sympathetic neurotransmitter or as a co-transmitter with NA, it has been difficult to describe its relative functional importance. Extensive investigation has shown that the role of ATP can be affected by different conditions, including the parameters used for EFS, the size of blood vessels studied and experimental conditions used. For example,

in rabbit saphenous artery, the responses were monophasic when the electrical stimulation period was for 1 s and became biphasic when the stimulation period was increased to 1 min. The initial fast phase was mediated by ATP, and this was followed by a second slow phase mediated by NA. These observations lead to the conclusion that the ratio of ATP to NA released was dependent upon the frequency of stimulation (Burnstock and Warland, 1987). Furthermore, it has been demonstrated that the purinergic component of responses is favoured by relatively short trains of stimuli at lower frequencies in small mesenteric arteries of rat (Sjoblom-Widfeldt and Nilsson, 1990).

The size of the blood vessels has been shown to affect the purinergic response. For example, in the rabbit jejunal branches of mesenteric artery and in the guinea-pig submucosal arterioles ATP has been shown to be the principal transmitter mediating contractile responses in response to EFS, with NA acting as a prejunctional neuromodulator through $\alpha_2$-adrenoceptors (Ramme et al., 1987, Evans and Surprenant, 1992). Furthermore, in rat mesenteric arteries the purinergic component becomes larger as the size of the arteries decrease (Gitterman and Evans, 2001).

Experimental conditions can also affect the role of ATP. For example, recent studies in rat mesenteric arteries have showed that electrically-evoked vasocontractile responses were mediated exclusively by NA under basal tone conditions, conversely, ATP contributed as functional neurotransmitter under more physiological conditions such as those produced by raising the tone with a vasoconstrictor agent (Pakdeechote et al., 2007). Furthermore, raising the pressure that arteries experience from 30 mmHg to 90 mmHg, which is close

to that experienced by rat mesenteric arteries in vivo, has shown that ATP become the dominant neurotransmitter mediating the electrically-evoked vasocontractile responses (Rummery et al., 2007).

#### 1.3.2.6 Purinoceptors

In 1978 Burnstock was the first to suggest a basis for distinguishing two types of purinergic receptors based on three factors, the relative potency to ATP, ADP, AMP and adenosine, the effectiveness of methylxanthines as antagonists, and finally by their ability to activate adenylate cyclase. Thus, according to this classification, receptors where adenosine was the principal natural ligand were termed P1 receptors while those receptors where the principal ligands were ATP and ADP were termed P2 receptors (Burnstock, 1978). Subsequently classifications of P2 receptors were made. P2 receptors were classified into P2X and P2Y receptors on the basis of the potency of structural analogues of ATP, such as α,β-methyleneATP and the ANPP33 (Burnstock and Kennedy, 1985).

Based on this information and on the basis of the molecular structure and cloned receptors P2 receptors, are subdivided into two families: ligand-gated ion channels mediating fast responses, P2X receptors, and G-protein-coupled receptors mediating slow responses, P2Y receptors (Abbracchio and Burnstock, 1994, Fredholm et al., 1994, Burnstock, 1996). Significant developments in molecular, biochemical and pharmacological techniques have led to the further subdivision of purinoceptors. Thus at present P2 receptors are divided into two groups, P2X and P2Y. P2X receptors are divided into $P2X_1$,

$P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors. The P2Y family is divided into $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors. P1 receptors are subdivided into $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ receptors (Ralevic and Burnstock, 1998, Alexander, 2009).

#### 1.3.2.6.1 P2X receptors

The P2X receptors are trimeric ATP-gated cation channels, which allow the entry of $Ca^{2+}$ in addition to monovalent cations such as $Na^+$ and $K^+$, and small organic cations (Benham and Tsien, 1987, Valera et al., 1994). Using radioligand assay with [$^3$H]α,β-methyleneATP, Bo and Burnstock provided direct evidence for the presence of P2X receptors on vascular smooth muscle (Bo and Burnstock, 1993). The use of antibodies against the extracellular domain of the P2X receptors and against the ubiquitous synaptic vesicle proteoglycan (SV2), showed the presence of P2X receptor clusters. Those were present on the smooth muscle cells of mesenteric, renal, and pulmonary arteries as well as in the aorta and in veins of rat (Hansen et al., 1999). In addition, the three-dimensional reconstruction of the P2X and SV2 labelling at individual varicosities using confocal microscopy, showed that the varicosities were located immediately apposite to the P2X receptor clusters (Hansen et al., 1999). Data obtained from mouse vas deferens support P2X clustering opposite the sympathetic varicosities where anti-P2X combined with anti-SV2 staining showed that P2X receptor clusters at single sympathetic varicosities (Barden et al., 1999). However, other studies have shown that P2X receptors are uniformly distributed around the circumference of each smooth muscle cell (no clusters), regardless of the existence or the absence of adjacent sympathetic

varicosity. For example, immunofluorescence staining of the vas deferens with antibodies against P2X receptor showed a diffuse distribution over the entire membrane of each smooth muscle cell that was not clustered beneath anti-SV2-stained sympathetic varicosities on smooth muscle cells of the mouse vas deferens (Liang et al., 2001).

In response to ATP, P2X receptors mediate vasoconstriction (Ralevic and Burnstock, 1998). In the vasculature it has been shown that P2X receptor activation mediates vasoconstriction in a number of blood vessels including rabbit basilar artery (Lee et al., 1976), rat pulmonary vessels (Liu et al., 1989) and renal arteries (Inscho et al., 1994). Furthermore, it was shown that the postjunctional responses of sympathetic nerve stimulation were solely mediated through the activation of P2X purinoceptors in submucosal arterioles of the guinea-pig (Evans and Surprenant, 1992). Moreover, in P2X-receptor-deficient mice, the P2X agonist α,β-methyleneATP failed to elicit any response indicating the absolute requirements of P2X receptors to mediate α,β-methyleneATP responses (Lamont et al., 2006).

With regard to the vasculature the $P2X_1$ receptor, which was cloned in 1994 (Valera et al., 1994), seems to be more involved in mediating responses to ATP as a neurotransmitter than other P2X receptor subtypes. Furthermore, in a study characterizing P2X receptor immunoreactivity, it was shown that $P2X_1$ receptors were expressed in a number of blood vessels including femoral, pulmonary, cerebral and renal arteries of the rat (Lewis and Evans, 2001). It also has been demonstrated, by immunohistochemical analysis, that the $P2X_1$ receptor is expressed at high levels in small and medium sized mesenteric

arteries from rats (Gitterman and Evans, 2000). Thus it seems, at least in rat vasculature, that $P2X_1$ receptors are expressed in almost all blood vessels which may be indicative of the relative importance of these receptors in the mediation of the purinergic response. In regard to human vasculature the presence of $P2X_1$ receptors has been shown using immunohistochemistry in a number of blood vessels. For example, $P2X_1$ receptors have been shown in postmortem human cerebral arteries (Bo et al., 1998a) and human umbilical vessels (Bo et al., 1998b). In addition, on circular and longitudinal smooth muscle of human long saphenous vein (Metcalfe et al., 2007).

$P2X_1$ receptors are also expressed on the endothelium of some human blood vessels including the internal mammary artery, radial artery and saphenous vein (Ray et al., 2002). It has been suggested that the function of $P2X_1$ receptors is dependent on its location, inducing contraction when located on smooth muscle cells, and dilation when expressed on the endothelium (Harrington and Mitchell, 2005).

However, the presence of other P2X receptors on endothelium has also been shown. For example, the presence of $P2X_2$ receptors in small cerebral arteries of the rat (Loesch and Burnstock, 2000). Furthermore, $P2X_4$ and $P2X_5$ receptors were expressed in human endothelial cell monolayers where it has been suggested that they are involved in self regulation of endothelial function and therefore modulating the vascular tone (Schwiebert et al., 2002). $P2X_4$ and $P2X_6$ receptors were also found on human endothelial cells where they have been shown to play a role in cell permeability and adhesion through their co-localisation with the cell adhesion molecule VE-cadherin (Glass et al., 2002).

Moreover, $P2X_4$ has also been shown on human vascular endothelial cells where their activation resulted in calcium influx (Yamamoto et al., 2000). Furthermore, in $P2X_4$ deficient mice, an impaired influx of $Ca^{2+}$ and subsequent production of NO has been reported. Thus, the ability of blood vessels to adapt to an acute increase in blood flow by vasodilatation or to adapt to chronic decrease in blood flow by vasoconstriction was impaired (Yamamoto et al., 2006).

At present, apart from the $P2X_6$ receptor, it seems that all P2X receptors are expressed in the splanchnic circulation at least in arteries, with a strong expression of $P2X_1$, $P2X_4$, $P2X_5$ and $P2X_7$ and weak expression of $P2X_2$ and $P2X_3$ (Phillips and Hill, 1999). However, even though they may all be present functional studies indicate a major role only for $P2X_1$ receptor in mediation of the purinergic neurogenic response (Lewis and Evans, 2001).

#### 1.3.2.6.2 P2Y receptors

P2Y receptors belong to the family of G-protein-coupled receptors (GPCRs)**.** On the basis of the action of their endogenous agonists, P2Y receptors can be divided into adenine nucleotide-preferring ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$) and uracil nucleotide-preferring ($P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$) receptors (von Kugelgen, 2006, Alexander, 2009). Furthermore, according to their G protein coupling and second messenger systems P2Y receptors can also be subdivided into two groups. One group includes $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors which are coupled primarily through members of the Gq/11 family of G proteins to activate phospholipase C, resulting in elevation of intracellular calcium levels. The other group includes $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ receptors,

which are coupled primarily to inhibition of adenylyl cyclase, resulting in a reduced accumulation of cyclic AMP (Ralevic, 2009).

Generally P2Y receptors are expressed on vascular endothelium where they mediate vasorelaxation to purines and pyrimidine nucleotides through generation of NO and EDHF (Ralevic, 2009). In regard to human blood vessels immunohistochemical staining showed the presence of $P2Y_2$ receptors on the endothelium of some human blood vessels including the internal mammary artery, radial artery and saphenous vein (Ray et al., 2002). Furthermore, immunoreactivity for $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors has been shown on the vascular endothelium of human umbilical vein (Wang et al., 2002). It seems that the most dominant functional P2Y receptors which are expressed on vascular endothelial cells are $P2Y_1$ (responding to ADP and ATP) and $P2Y_2$ (similarly sensitive to UTP and ATP) where they act as sensors for shear stress and hypoxia in response to locally released purines (Ralevic, 2009).

However, P2Y receptors can also be found on vascular smooth muscle. For example, immunoreactivity to $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors was demonstrated on circular and longitudinal smooth muscle of human long saphenous vein (Metcalfe et al., 2007). Functional studies have shown evidence for the involvement mainly of $P2Y_2$ and $P2Y_6$ in regulation of blood vessels contractility. For example, $P2Y_2$ receptors have been shown to be involved in the mediation of contractile responses to uracil nucleotides in human coronary arteries (Malmsjo et al., 2000). In addition, $P2Y_2$ and $P2Y_6$ receptors have also been shown to be involved in the mediation of contractile

responses to uracil nucleotides in rat cerebral microvasculature (Lewis et al., 2000).

#### 1.3.2.7 ATP regulation of vascular tone

ATP may be not only found in sympathetic nerves but also in sensory nerves (Burnstock and Ralevic, 1994). In addition to perivascular nerves, ATP can be released from other sources including endothelium, blood borne cells (RBC and platelets), damaged tissues and smooth muscle cells (Burnstock, 2008, Ralevic, 2009). The neuronally released ATP and ATP released from other sources are degraded by ectonucleotidases into ADP, AMP and adenosine (Todorov et al., 1997, Zimmermann, 2006). Therefore, ATP released from different sources can be degraded into ADP and adenosine thus providing a source for these active substances that have been shown to be involved in the regulation of vascular tone. For example, it has been shown that ADP evoked contractions in human internal mammary artery through the activation of $P2Y_{12}$ receptors (Wihlborg et al., 2004). It has also been demonstrated that ATP and its degradation product adenosine mediated negative feedback by ATP acting on P2Y receptors and adenosine acting on P1 ($A_1$) receptors in sympathetic nerves (Boehm and Huck, 1997, Cunha, 2001). Therefore, ATP acts directly as a neuronally released neurotransmitter via P2X and some P2Y receptors and through its products of degradation (ADP and adenosine) acting on different P2Y and P1 receptors, and as an extracellular signalling molecule released from endothelium and other tissues can influence different systems and organs in the human body (see Fig. 1.2). ATP effects mediated through its direct involvement or via its products of breakdown have been shown in recent

studies. For example, a role of ATP has been shown in the pathophysiology of hypertension (Inscho et al., 2004), pulmonary hypertension, (Sprague et al., 2003) and atherosclerosis (Burnstock, 2002, Gerasimovskaya et al., 2008).

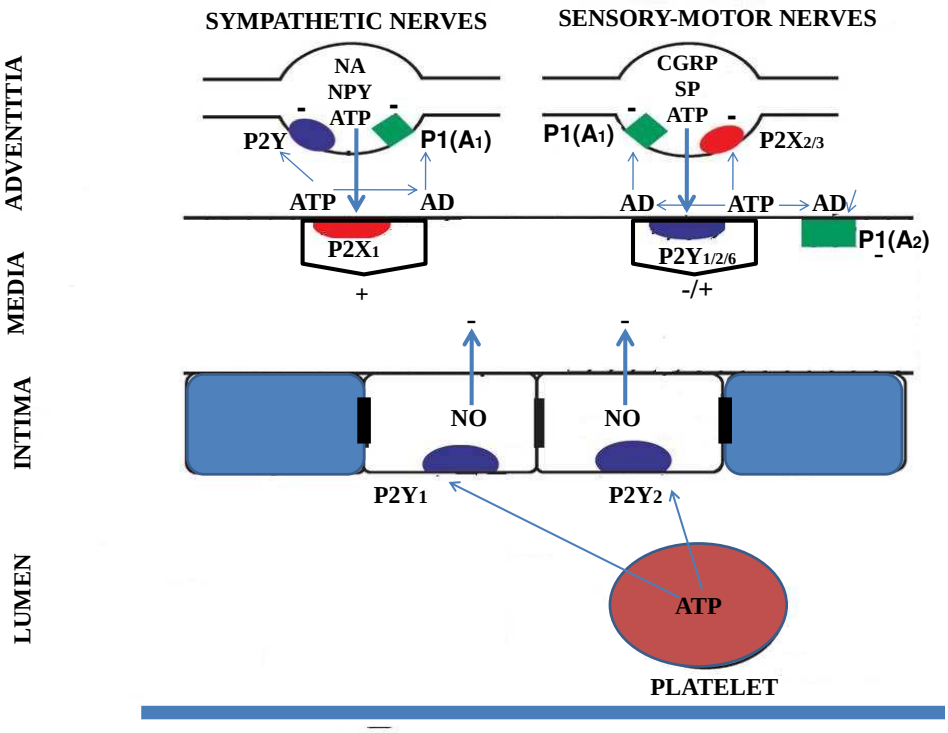



Fig. 1.2 Diagram shows the effects of different purines involved in the control of blood vessels. ATP is released as a cotransmitter with noradrenaline (NA) and neuropeptide Y (NPY) from sympathetic nerves in the adventitia to act at smooth muscle $P2X_1$ receptors, causing constriction; ATP is released with calcitonin gene related peptide (CGRP) and substance P (SP) from sensory-motor nerves to act on smooth muscle P2Y receptors, causing vasodilatation. P1 ($A_1$) receptors on nerve terminals of sympathetic and sensory nerves mediate adenosine's (originating from the breakdown of ATP) modulation of transmitter release. $P2X_{2/3}$ receptors are present on sensory nerve endings. P1 ($A_2$) receptors on vascular smooth muscle mediate vasodilatation. Aggregating platelets release ATP to act on endothelial $P2Y_1$ and $P2Y_2$ receptors causing the release of nitric oxide (NO) and subsequent vasodilatation. (Adapted from Burnstock 2008).

### 1.3.3 NEUROPEPTIDE Y (NPY)

Using the technique of high performance liquid chromatography examining samples obtained from porcine brain, a new peptide was discovered that was called neuropeptide Y (NPY) (Tatemoto et al., 1982a, Tatemoto, 1982b). In 1983, Lundberg and co-workers used radioimmunoassay and immunocytochemistry techniques to show the presence of NPY in high levels in peripheral noradrenergic neurons in vas deferens and blood vessels of guinea-pig, cat, pig and man (Lundberg et al., 1983). Chemical and surgical sympathectomy followed by immunohistochemical investigation have demonstrated the coexistence and cooperation of NA and NPY in perivascular nerves of many blood vessels in mice, rat, guinea-pig, rabbits and humans (Ekblad et al., 1984). Furthermore, in the rat vas deferens NPY and NA have been located in large vesicles while the small vesicles contain NA (Fried et al., 1985a, Fried et al., 1985b).

The presence of NPY in sympathetic vesicles co-stored with NA poses questions about its physiological role in the effector organs supplied by sympathetic nerves, such as blood vessels. The exogenous application of NPY on isolated segments of basilar, gastro-epiploic, and femoral arteries in the rabbit as well as in smooth muscle of cerebral arteries of cats, potentiated the vasoconstrictor effect of NA (Edvinsson et al., 1984, Edvinsson, 1985). Furthermore, a recent study using the wire myograph coupled with confocal microscopy showed that exogenous NPY increased calcium waves frequency produced by the noradrenergic agonist phenylephrine in rat mesenteric arteries (Wier et al., 2009). Other experiments in the pig hind limb vasculature showed

that NPY played an important role as a sympathetic transmitter (Modin et al., 1993). The use of BIBP-3226, a $Y_1$ receptor selective antagonist, enabled Han and co-workers to demonstrate that 30% of the vasoconstriction produced by sympathetic nerve stimulation in the rat mesenteric vascular bed was mediated by NPY (Han et al., 1998).

With regard to vascular sympathetic neurotransmission, NPY acts on two NPY receptors, $NPY_1$ and $NPY_2$ (Wahlestedt et al., 1990). The $NPY_1$ receptor is located mostly postjunctionally on the surface of the vascular smooth muscle. It has been suggested that the $NPY_1$ receptor is mainly involved in potentiating the NA and ATP-evoked vasomotor responses in different blood vessels such as in the rat mesenteric bed (Donoso et al., 1997). The $NPY_2$ receptor is largely presynaptic in origin, mainly involved in reducing the release of NA and ATP from sympathetic perivascular nerves (see Fig. 1.3) (Pablo Huidobro-Toro and Verónica Donoso, 2004). At present there are five subtypes of NPY receptors: $NPY_1$, $NPY_2$, $NPY_4$, $NPY_5$ and $NPY_6$ (Alexander, 2009).

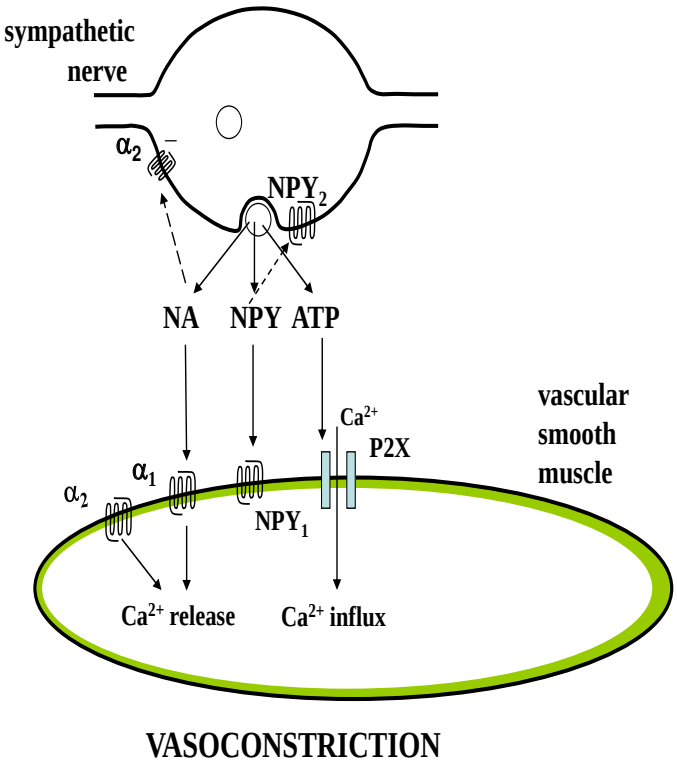



Figure. 1.3 Diagram shows the release of different neurotransmitters; noradrenaline (NA), ATP and neuropeptide Y (NPY) from sympathetic nerve varicosity, and their effect on different receptors; $\alpha_1$- and $\alpha_2$- adrenoceptors, $Y_1$ and $Y_2$ and P2X on the postsynaptic membrane. (Adapted from Ralevic 2009)

## 1.4 AIMS AND OBJECTIVES

The relative contribution of ATP as a sympathetic cotransmitter varies according to vascular preparation, size of blood vessels and the underlying experimental conditions such as vascular tone. Vascular tone is the ability of all blood vessels to exhibit a degree of constriction relative to their maximally dilated state under basal tone conditions in vivo. It has previously been shown that, in rat perfused mesenteric bed, raising vascular tone with U46619 revealed a functional role for ATP, while increasing intraluminal pressure showed a predominant functional role for ATP in pressurised rat mesenteric arteries. Thus, the aim was to investigate this in porcine tissue since it is more closely related to that of man.

# CHAPTER 2

# METHODS

## 2.1 PORCINE PERFUSED MESENTERIC ARTERIAL BED PREPARATION

Mesenteries were obtained from male or female large white Danish pigs, aged about 10 weeks and about 80 kilograms in weight, after slaughter at a local abattoir. The mesenteries (which had been separated from the intestines) were immediately transported to the laboratory in containers filled with chilled Krebs-Henseleit solution with the following composition (mM): NaCl 128, KCl 4.8, $NaHCO_3$ 25, $MgSO_4$ 1.1, $KH_2PO_4$ 1.2, $CaCl_2 2H_2O$ 1.25 and glucose 12, that had previously been gassed with 95% $O_2$–5% $CO_2$. The superior mesenteric artery was identified and one of the first order branches was dissected out together with part of the mesentery containing the whole vascular tree (i.e. the first order branch artery and the rest of its vascular tree) (see Fig. 2.1).

Tissue was then placed in Krebs solution containing 2% Ficoll and refrigerated overnight at 4 °C (preliminary experiments showed that the responses to EFS obtained in fresh tissues and in overnight incubated tissues were similar in size). The next day fine dissection was used to expose the first order branch artery, which was cannulated using a blunted hypodermic needle (No. 21). This acted as the site of perfusion and as the positive electrode. The cannulated preparation was then placed on a stainless steel grid (which acted as the negative electrode) in a humid chamber, in which it was perfused at a flow rate

of 5 ml / min using a peristaltic pump (model 755-30, Cole-Parmer, Chicago, IL) (see Fig. 2.2).

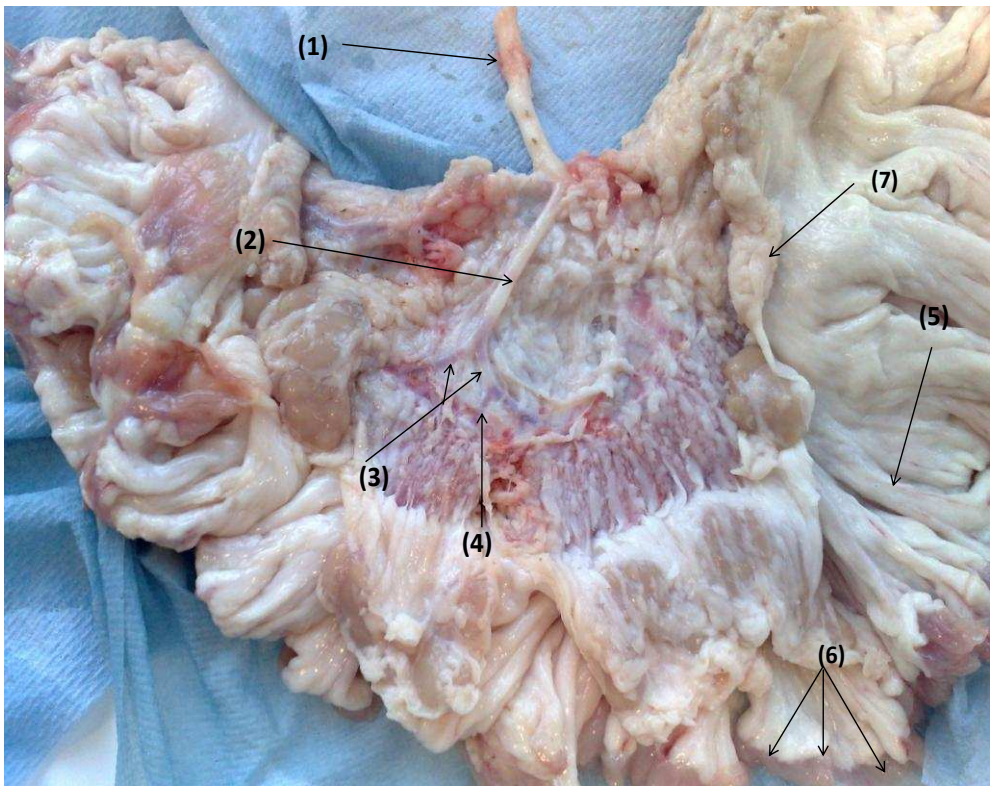



Fig. 2.1 Picture of the whole porcine isolated mesenteric arterial bed shows (1) superior mesenteric artery, (2) first order branch artery, (3) second order branch arteries, (4) third order branch artery, (5) terminal small arteries, (6) small intestine and (7) part of the tissue covering the mesenteric bed, which has been dissected and reflected to expose the underlying vascular tree.

In order to monitor changes in contractility of the mesenteric bed, a pressure transducer (model P23XL: Viggo-Spectramed, Oxnard, CA) was used to monitor the perfusion pressure (mmHg). Changes were recorded using a powerlab (ADInstruments, Pty Ltd., Castle Hill, Australia). The tissue was

equilibrated for 60 min before experimentation. A Grass SD9 stimulator was used to apply electrical field stimulation (EFS) (2-16 Hz, 1 ms, 90 V, 30 s).

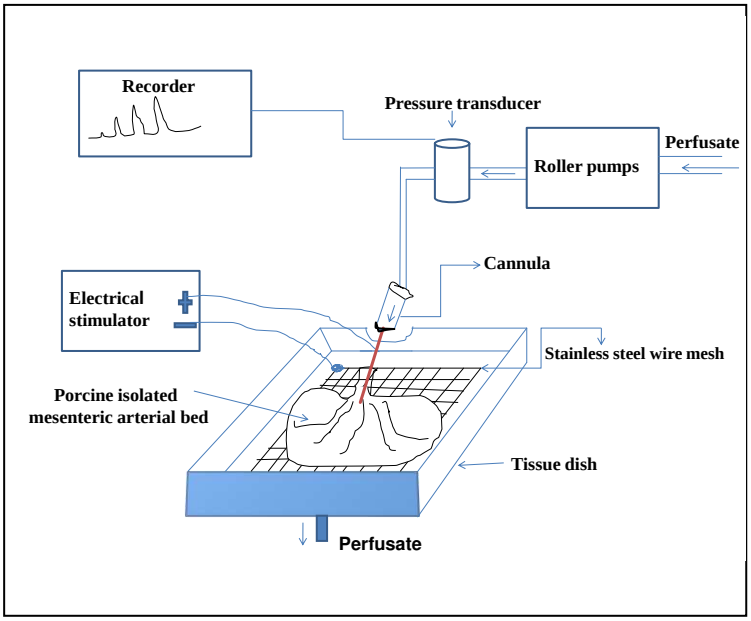



Fig. 2.2 Set up used to perfuse porcine isolated mesenteric arterial bed (see text for details).

## 2.2 PREPARATION OF PORCINE ISOLATED ARTERIES FOR ISOMETRIC RECORDING

Porcine mesenteries were obtained from a local abattoir and transported to the laboratory in Krebs-Henseleit buffer placed on ice. First and third order porcine mesenteric arteries were dissected out and placed in Krebs-Henseleit buffer with the following composition (mM) NaCl 128, KCl 4.8, $NaHCO_3$ 25, $MgSO_4$ 1.1, $KH_2PO_4$ 1.2, $CaCl_2 2H_2O$ 1.25 and glucose 12, gassed with 95% $O_2$–5% $CO_2$ containing 2% Ficoll and stored overnight at 4 ºC. The next day mesenteric arteries were dissected into 5 mm segments and suspended between two supports, in organ baths containing Krebs-Henseleit buffer maintained at 37 ºC and constantly gassed with 95% $O_2$–5% $CO_2$. A thin wire (upper support) was inserted through the lumen of the arterial ring while a second

wire (lower support), attached to an electrode assembly, was also threaded through the lumen. The tissue was then suspended between the two supports (see Fig. 2.3). The electrode assembly was connected to a Grass SD9 stimulator, while the upper support was connected by a thread to a force transducer (World Precision Instruments, Sarasota, Florida, U.S.A) linked to a Maclab data acquisition system (AD Instruments Ltd, Hastings, UK) via an amplifier. After a 15 min equilibration period, tension was applied to the first order arteries (10 g) and to the third order arteries (4 g). Arteries were allowed to relax to a final resting tension of between (2-4 g) for first order arteries and (1-2 g) for third order arteries. The tissues were contracted 2 times with KCl (60 mM) with a 30 min interval between the contractions. Tissues were then washed with Krebs-Henseleit buffer and allowed to equilibrate for 60 min. After the equilibration period, responses to EFS (2-32 Hz, 1ms, 90 V, 30 s) were determined. The interval between the frequencies was variable (2-5 min), and was determined by the return to baseline after each stimulation, with a minimum interval of 2 min if there was no response. In other experiments responses to α,β-methyleneATP and exogenous NA were determined.

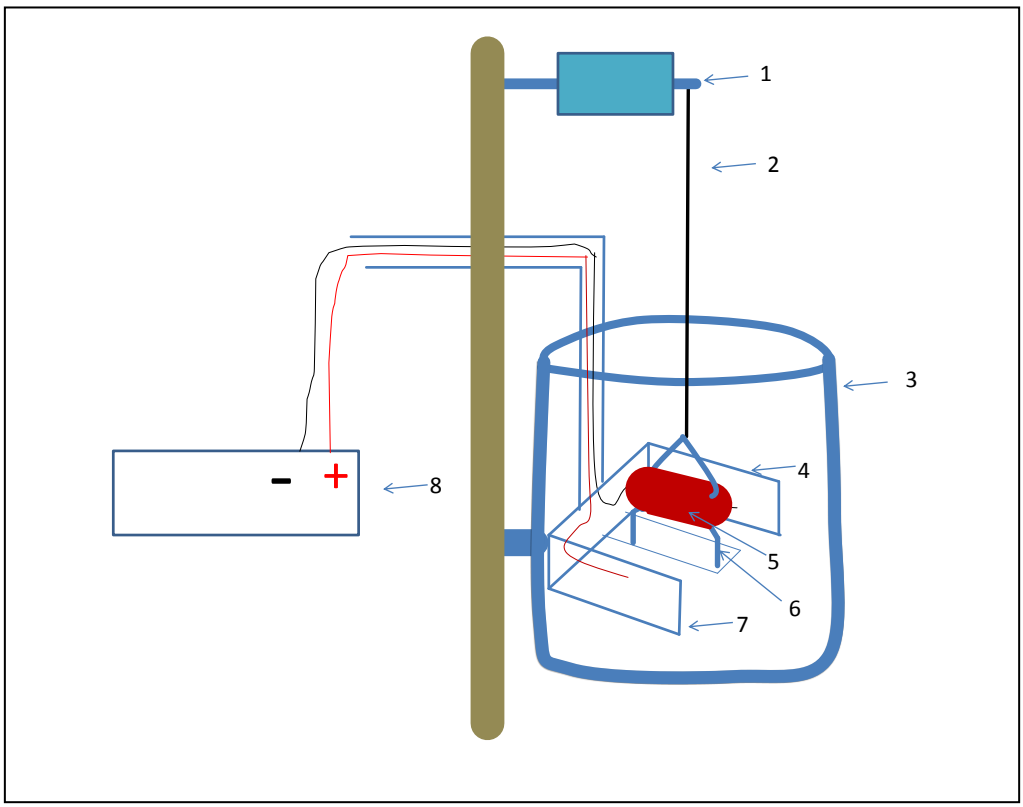



Fig. 2.3 Diagram showing an isometric set up including (1) transducer, (2) a thin thread attached to a thin wire (upper support), (3) organ bath, (4) negative electrode, (5) blood vessel mounted between upper and lower supports, (6) thin wire (lower support) attached to a tissue holder, (7) positive electrode, (8) electrical stimulator.

## 2.3 IMMUNOHISTOCHEMICAL STAINING

### 2.3.1 Tissue preparation

Porcine mesenteries were obtained as described in section 2.1. Fine dissection was carried out to identify small terminal branch arteries using a dissecting microscope (see Fig. 2.1). Arteries were carefully dissected free of excess fat and connective tissue and cut into segments approximately 5 mm long. For immunohistochemical staining, segments were fixed in 4% paraformaldehyde,

for 2 h. Vessels were then placed in a 24 well plate where 5 ml of phosphate buffered saline (PBS) was added onto each specimen for 30 min. Specimens were washed in phosphate-buffered saline twice for 5 min, after which they were ready for staining.

### 2.3.2 Immunohistochemical Staining

Whole-mount segments of porcine mesenteric arteries were stained using the standard indirect immunofluorescence technique. Background staining was reduced by incubation in 2% normal goat serum (1 in 10 dilution) for 10 min at room temperature, in all preparations. In some preparations primary mouse monoclonal antibody to protein gene product 9.5 (PGP9.5) (1 in 10 dilution) was applied for 24 h in a humid chamber at room temperature. After washing twice for 5 min in 0.2% Tween in phosphate-buffered saline, fluoresceine conjugated rabbit anti–mouse IgG secondary antibody (Alexa Fluor 568 bright orange-red fluorescent dye) was applied for 2 h in a humid chamber covered with foil at room temperature. In other preparations primary rabbit polyclonal antibody to tyrosine hydroxylase (TH) (1 in 500 dilution), was applied for 24 h in a humid chamber at room temperature. Tissues were washed twice for 5 min in 0.2% Tween in phosphate-buffered saline, then fluoresceine conjugated goat anti–rabbit IgG secondary antibody (Alexa Fluor 488 bright green fluorescent dye) was applied for 2 h in a humid chamber covered with foil at room temperature. All preparations were washed a further 2 times for 5 min in 0.2% Tween in phosphate-buffered saline, then DAPI (1 in 500 dilution), a fluorescent nuclear stain in 0.2% Tween in phosphate-buffered saline was added to the samples for 10 min. After a further 2 times washing for 5 min in

0.2% Tween in phosphate-buffered saline the samples were mounted on glass slides. In parallel, in some arteries, the same protocol was used without the addition of primary antibodies as controls.

Fluorescence was detected using a Leica TCS SP2 confocal laser scanning microscope equipped with 405 diode, argon and 561 lasers. DAPI, Alexa 488, and Alexa 568 were excited with the 405, 488 and 561 nm lines respectively. Each arterial segment was imaged separately, emissions were separated using an acousto-optical beam splitter (AOBS), and then merged using the Leica confocal software. Whole mount preparations were viewed using a Leica DMIRE2 stand and a 20X plan apochromat objective with a 0.7 numerical aperture. The images (512 x 512 pixels) were then obtained sequentially from two channels using a confocal pinhole of 1. The images were stored as TIFF files. Z-stacks were acquired using a 1.5 µm step height. Each frame was acquired 3 times and an average presented.

## 2.4 PORCINE MESENTERIC SMALL ARTERIES PREPARATION

Porcine mesenteries were obtained as described in section 2.1. Fine dissection was carried out to identify the small terminal branch arteries (see Fig. 2.1) using a dissecting microscope. Small arteries were carefully dissected free of excess fat, connective tissue and separated from the veins and then cut into segments approximately 2 µm long. Next, vessels were mounted on fine tungsten wires (40 µm diameter), and placed between the plastic jaws of a dual channel Mulvany-Halpern wire myograph (Myo-Interface Model 410A,

Danish Myo Technology, Denmark) (Mulvany and Halpern, 1977) (see Fig. 2.4). Vessels were kept at 37°C in Krebs–Henseleit buffer, gassed with 5% $CO_2$/95% $O_2$. A tension of 1g was applied (preliminary experiments showed that when 1 g tension was applied, a larger response was obtained compared to that produced when 0.5 g was applied to the mounted vessels). Vessels were left to relax to a resting tension of 0.3- 0.5 g. The viability and contractile integrity of each vessel was then tested by its ability to contract to KCl (60 mM) by at least 0.4 g. Tension was measured and was recorded on a MacLab 4e recording system (ADInstruments, U.K.).

The perivascular nerves were activated electrically through two platinum electrodes mounted in the plastic jaws, either side of the blood vessel. EFS (2-16 Hz, 1 ms, 10 V, 30 s) was supplied by a stimulator unit (DS2; Digitimer Ltd, Welwyn Garden City, UK).

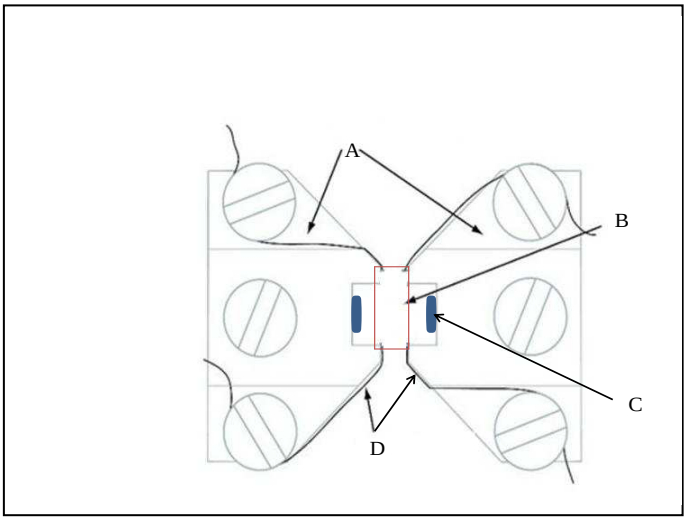



Fig. 2.4 Diagram showing mounting apparatus of a wire myograph including A) mounting plastic jaws, B) mounted blood vessel, C) stimulation electrode and D) fine tungsten wires (40 µm diameter).

## 2.5 RAT MESENTERIC ARTERIES PREPARATION

Male Wistar rats (200–250 g) were stunned by a blow to the cranium and killed by exsanguination. The gut with attached mesenteric vascular bed was removed through an incision in the abdominal cavity. The mesentery was then kept in physiological salt solution containing (mM): NaCl, 118; $NaHCO_3$, 25; KCl,4.8; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; glucose, 11.1; $CaCl_2$, 1.25; and gassed with 95% $O_2$–5% $CO_2$ (pH 7.4). Thereafter, a first order mesenteric artery was identified and followed to the point where it further divided into second-order branches and followed until they further divided into third-order branches. This part of the vascular tree was isolated, and adherent fat and connective tissue were removed from the first and the second order arteries, except at the branch point, where it was left intact. The pre-dissected tissue was then transferred into a pressure myograph filled with physiological salt solution. The pressure myograph contains two electrodes; on one end a glass electrode and on the other end a suction electrode. Next, using a dissecting microscope, the first order artery was directed towards the suction electrode and sucked into it (see Fig. 2.5). Meanwhile the second order artery was directed towards the glass electrode and mounted onto it and tied securely using a fine nylon suture. Thereafter, physiological salt solution was flushed through the glass electrode to remove any remaining blood in the second order artery. Finally the second order mesenteric artery was tied off just distal to the branch point and the fat left intact at the branch point was then used to create a tight seal at the mouth of the suction electrode. The glass cannula, which was filled with physiological saline, was then connected to a pressure servo controlled

peristaltic pump (Living Systems, Burlington, VT, USA), allowing precise control of intraluminal pressure. The tissues were superfused with physiological salt solution and maintained at 36–37˚C. Vessels were pressurized to 90 mmHg, and allowed to equilibrate for 20 – 30 min.

The perivascular nerves were activated electrically through a stimulating electrode that consisted of a polyethylene tube (~400 μm lumen diameter) with a silver wire electrode within the lumen and another electrode wrapped around the outside. EFS (1 ms, 50 pulses, 10-20 V, 0.5-10 Hz) was supplied by a stimulus isolation unit (DS2; Digitimer Ltd, Welwyn Garden City, UK). Arteries were visualized using a CCD camera attached to an inverted microscope and their inner diameter was continually monitored using an edge tracking device (video dimension analyser model V94; Living Systems, Burlington, VT, USA).

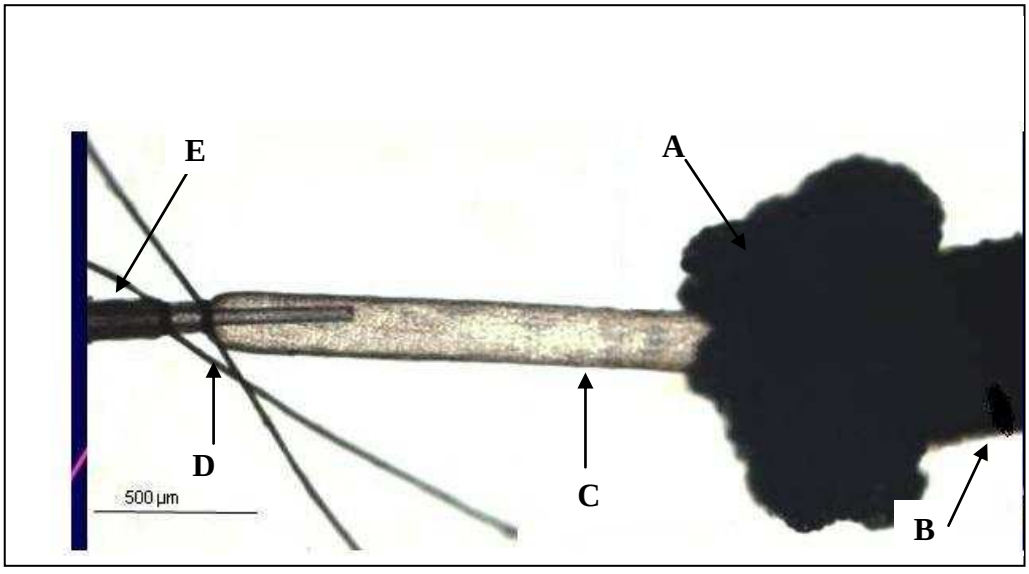



Fig. 2.5 Set up for a pressure myograph showing (A) fat tissue left to form tight seal, (B) suction electrode, (C) second order rat mesenteric artery pressurised to 90 mmHg, (D) thread to tie the artery and (E) glass cannula connected to a pressure transducer.

## 2.6 PORCINE MESENTERIC ARTERIES PREPARATION

Porcine mesenteries were obtained as described in section 2.1. Fine dissection was carried out to identify the small terminal branch arteries using a dissecting microscope. The anatomical pattern of the porcine small blood vessels was different from that of the rat. There was no obvious branching pattern although there were long segments of small arteries that were companied by a closely attached vein. Therefore, these vessels were carefully dissected free of excess fat and connective tissue and then separated from the veins as follows. Three quarters of the closely attached vein were carefully dissected and removed while the remaining piece of the vein and its surrounding connective tissue was left (to enable creation of a tight seal in the suction electrode). The pre-dissected tissue was transferred to the pressure myograph chamber which was filled with physiological salt solution. Using a dissecting microscope, the end of the artery that was left with the remaining piece of the vein and its surrounding connective tissue attached was directed towards the suction electrode and sucked into it. Meanwhile the other end of the artery was directed towards the glass electrode and mounted onto it, with the electrode in the lumen of the vessel, and tied securely using fine nylon suture. Thereafter, physiological salt solution was flushed through the glass electrode to remove any remaining blood in the artery. Finally the end of the artery that was left intact with its surrounding connective tissue and the vein was tied off, and sucked into the suction electrode to create a tight seal at the mouth of the suction electrode.

## 2.7 DRUGS

Prazosin, α,β-methyleneATP, guanethidine, nifedipine, U46619 and capsaicin were obtained from Sigma (Poole, Dorset, UK). BIBP3226 and endothelin-1 (porcine) were obtained from TOCRIS, Bristol USA. RX811059 was from Reckit and Colman, Hull, UK. (±) arterenol (noradrenaline hydrochloride) and YM-12617 were obtained from Sigma (Poole, Dorset, UK). NF-449 was obtained from Calbiochem, Nottingham, UK. U46619 stock concentration was 10 mg/ml in methyl acetate and reconstituted in ethanol to form a $10^{-2}$ M solution, while capsaicin and nifedipine were dissolved in ethanol to form a $10^{-1}$ M solution for capsaicin and $10^{-3}$ M for nifedipine. All other drugs were dissolved in water. The experiments with NF-449 and nifedipine were performed with the light in the laboratory switched off due to the light sensitivity of the compound.

# CHAPTER 3

# ATP ACTS AS A FUNCTIONAL SYMPATHETIC NEUROTRANSMITTER IN THE PORCINE PERFUSED MESENTERY AFTER RAISING TONE

## 3.1 INTRODUCTION

Prior to 1970 NA was considered to be the sole neurotransmitter released from sympathetic perivascular nerves in blood vessels. However, the establishment of co-transmission as a concept in autonomic neuroscience (Burnstock, 1976, Burnstock, 2004) made it possible for other biological substances to be considered as neurotransmitters released with NA from the sympathetic perivascular nerves. For example, ATP has now been shown to be released from sympathetic nerves as a co-transmitter in a variety of different blood vessels, including the rabbit mesenteric artery (Ishikawa, 1985), rabbit hepatic artery (Brizzolara and Burnstock, 1990) and human saphenous vein (Racchi et al., 1999).

A number of studies have investigated the importance of ATP as a sympathetic neurotransmitter, mainly using isolated arteries, and it has been shown that the role for ATP as a sympathetic neurotransmitter is more evident in small arteries compared to large (Martin et al., 1991, Gitterman and Evans, 2001). Relatively few studies have investigated the role of ATP as a neurotransmitter in the whole organ. However, in the rat isolated perfused kidney, stimulation of periarterial nerves at lower frequencies produced renal vasoconstriction that was mediated by ATP (Schwartz and Malik, 1989). In a different study in the

same bed ATP was shown to act as neuromodulator where, after it was released from sympathetic nerves, it inhibited the release of NA, via stimulation of prejunctional P2 receptors (Bohmann et al., 1997). In the rat isolated mesenteric arterial bed responses to nerve stimulation did not involve ATP under normal experimental conditions. However, after raising tone (pre-constriction of the blood vessels) with vasoconstrictor agents including (U46619 a thromboxane $A_2$ agonist) or endothelin-1, ATP was shown to be involved as a functional sympathetic neurotransmitter (Pakdeechote et al., 2007).

In the present study, I have used the mesenteric arterial bed isolated from pigs to investigate the potential involvement of ATP as a sympathetic neurotransmitter. The mesenteric vascular bed plays a very important role in determining the haemodynamics of the cardiovascular system, with up to 25 % of blood flow passing through mesenteric arteries (Takagi et al., 1988) and the porcine cardiovascular system is similar in terms of haemodynamics and anatomical size to the human cardiovascular system. U46619 was used to increase the baseline perfusion pressure.

## 3.2 Materials and methods

### 3.2.1 Porcine perfused mesenteric arterial bed preparation

Mesenteries were obtained from male or female large white Danish pigs, after slaughter at a local abattoir. The mesenteries were immediately transported to

the laboratory in containers filled with chilled Krebs-Henseleit solution. The mesenteric arterial bed was set up as described in section 2.1.

### 3.2.2 Responses to EFS in porcine isolated mesenteric arterial bed under basal tone conditions

After an initial 60 min equilibration period, responses to EFS (2-16 Hz, 1 ms, 90 V, 30 s) were determined. The interval between the frequencies was variable (2-10 min), and was determined by the return to baseline after each stimulation, with longer intervals at higher frequencies. After a further 30 min, a second frequency response curve (FRC) was generated to investigate the reproducibility of the FRCs. In some experiments prazosin (0.1 μM), an $\alpha_1$-adrenoceptor antagonist (Cambridge et al., 1977, Pakdeechote et al., 2007), was added after the first FRC, while a combination of prazosin (0.1 μM) plus guanethidine (1 μM), a sympathetic neurone blocking agent (Williams and Clarke, 1995), was added after the second FRC and a third FRC was constructed. In other experiments α,β-methyleneATP (1 μM) (Kasakov and Burnstock, 1982, Sneddon and Burnstock, 1984, Neild and Kotecha, 1986) was added after the first FRC. α,β-methyleneATP is a P2X receptor desensitizing agent which acts at two different receptors $P2X_1$ and $P2X_3$ receptors; $P2X_1$ receptors are expressed on the vascular smooth muscle where they cause vasoconstriction, while $P2X_3$ receptors are expressed mainly on sensory nerves (Ralevic and Burnstock, 1998). All antagonist drugs were left for 30 min before testing their effect on the electrically-evoked vasocontractile responses.

### 3.2.3 Responses to EFS in porcine isolated mesenteric arterial bed under raised tone conditions

U46619, a thromboxane $A_2$ agonist (10-50 nM), was added to raise the tone in each preparation (25 ± 10 mmHg above baseline) (Pakdeechote et al., 2007), before generating responses to EFS. In some preparations a FRC was obtained under basal tone conditions then U46619 was added to raise the tone and a second FRC was conducted. In different preparations, after raising tone, two consecutive FRCs were generated, separated by a 30 min interval. These were used to investigate the reproducibility of the FRCs. In separate preparations the effects of (a) guanethidine (1 μM), (b) prazosin (0.1 μM), (c) α,β-methyleneATP (1 μM), and (d) α,β-methyleneATP (1 μM) plus prazosin (0.1 μM) were investigated by adding these after the first FRC. In some preparations a FRC was generated in the presence of U46619 and the tissue was then incubated with capsaicin (1 μM), for 30 min. The tissue was then washed with Krebs-Henseleit buffer for 30 min and re-exposed to U46619 before a second FRC was obtained. All antagonist drugs were left for 30 min before testing their effect on the electrically-evoked vasocontractile responses.

## 3.3 STATISTICAL ANALYSIS

Data were expressed as a percentage of the response obtained at 16 Hz of the first frequency response curve. Results are expressed as the mean ± S.E.M. Statistical comparisons were made by two way analysis of variance (ANOVA) with Bonferroni post-hoc test, or Student's t-test. A value of $P < 0.05$ was taken to indicate statistical significance.

## 3.4 RESULTS

### 3.4.1 Role of $\alpha_1$-adrenoceptors and P2X receptors in mediating electrically-evoked vasocontractile responses in porcine isolated mesenteric arterial bed under basal tone conditions

Under basal tone conditions (baseline pressure 42 ± 4 mmHg, n=14), EFS elicited frequency-dependent vasoconstrictor responses in the porcine isolated mesenteric arterial bed. These responses were relatively variable in amplitude, between preparations. Thus, responses were expressed as a percentage of the response to 16 Hz obtained in the first FRC. Under basal tone conditions two consecutive FRC were reproducible (n=9) (Fig. 3.1). Prazosin (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, significantly attenuated the electrically-evoked vasocontractile responses (e.g. by 70 ± 8% at 16 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 3.2). A small residual response remained that was not blocked by further addition of guanethidine (1 μM), a sympathetic neuron blocker, presumably due to direct smooth muscle activation. Neither prazosin nor guanethidine altered the basal perfusion pressure. α,β-methyleneATP (1 μM), a P2X receptor desensitising agent, produced a transient contraction (23 ± 4 mmHg, n=9) and its effect on responses was estimated once the perfusion pressure had returned to its original level, usually after 5-15 min. α,β-methyleneATP had no significant effect on the vasoconstrictor responses obtained to EFS in the porcine isolated mesenteric arterial bed under basal tone conditions (e.g. the response at 16 Hz

was 92 ± 20% of the control) (n=9) (Fig. 3.3A and Fig. 3.3B representative trace).

### 3.4.2 Role of $\alpha_1$-adrenoceptors and P2X receptors in mediating electrically-evoked vasocontractile responses in porcine mesenteric arterial bed under raised tone conditions

The thromboxane $A_2$ agonist U46619 (10-50 nM) increased the perfusion pressure from 42 ± 4 mmHg to 68 ± 7 mmHg ($P < 0.05$, Student's unpaired t-test) (n=14), and significantly enhanced the electrically-evoked vasocontractile response, particularly at higher frequencies (e.g. the response was increased at 16 Hz by 236 ± 75% ($P < 0.05$, ANOVA) (n=8) (Fig. 3.4A and Fig. 3.4B representative trace). In the presence of U46619, two consecutive FRCs were reproducible (n=5) (Fig. 3.5). Under these conditions guanethidine (1 µM) almost abolished the vasoconstrictor responses evoked by EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=4) (Fig. 3.6A). Prazosin (0.1 µM) did not alter the raised perfusion pressure but attenuated the electrically-evoked vasocontractile responses at all frequencies reaching significance at higher frequencies (e.g. 45 ± 5% inhibition at 16 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 3.6B) under raised tone conditions. The inhibition produced by prazosin in the presence of U46619 was much less than the 70% inhibition produced under basal tone conditions (see Fig. 3.2 and 3.6B).

In the presence of U46619, α,β-methyleneATP (1 µM) produced a transient contraction (114 ± 19 mmHg, n=10). This transient contraction was much

larger compared to that under basal tone conditions ($P < 0.001$, unpaired t-test, n=10) (see Fig. 3.3B and Fig. 3.7B). α,β-methyleneATP pre-treatment attenuated the vasoconstrictor responses obtained after EFS in the porcine isolated mesenteric arterial bed under conditions of raised tone at all frequencies, reaching statistical significance at 16 Hz (49 ± 9% inhibition, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 3.7A and Fig. 3.7B representative trace).

The combination of α,β-methyleneATP (1 μM) and prazosin (0.1 μM) together attenuated the responses at all frequencies of stimulation, reaching statistical significance at higher frequencies. The combination of α,β-methyleneATP and prazosin together had more effect than prazosin or α,β-methyleneATP had when they were added alone especially at higher frequencies (e.g. 85 ± 9% inhibition at 8 Hz, $P < 0.01$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 3.8) compared to the inhibition caused by either prazosin alone or by α,β-methyleneATP alone at 8 Hz (50% and 25% inhibition) respectively. However, the combination of α,β-methyleneATP and prazosin together had less effect compared to that of guanethidine especially at 16 Hz where guanethidine inhibited the response by about 85%.

### 3.4.3 Effects of capsaicin on the electrically-evoked vasocontractile responses in porcine mesenteric vascular bed under raised tone conditions

In the rat perfused mesenteric vascular bed, pre-treatment with capsaicin (1 μM), a TRPV1 receptor agonist, enhanced the electrically-evoked

vasocontractile responses (Pakdeechote et al., 2007). In the present study we investigated the effect of pre-treatment of the porcine arterial bed with capsaicin (1 μM) for 30 min. Capsaicin did not alter the basal or raised perfusion pressure and had no significant effect on the electrically-evoked vasocontractile responses in the porcine isolated mesenteric arterial bed (n=9) (Fig.3.9)

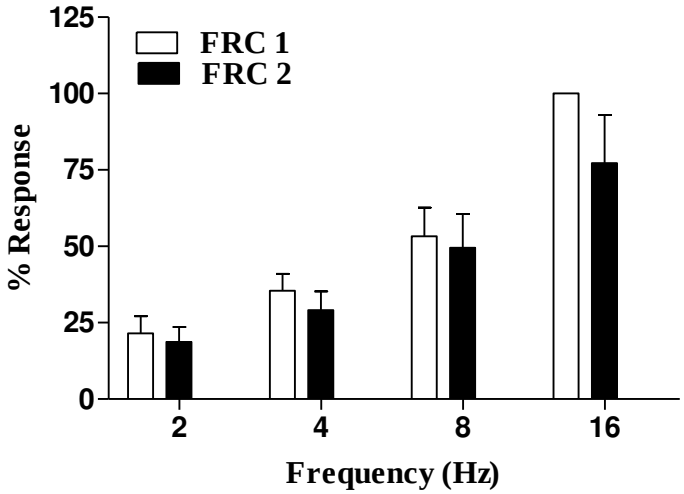



Fig. 3.1 Reproducibility of vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1ms, 30s, 90 V). Opened bars show the first frequency response curve (FRC 1) while closed bars show second frequency response curve (FRC 2) in the porcine isolated mesenteric arterial bed under basal tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve (n=9). Data are shown as mean ± standard error.

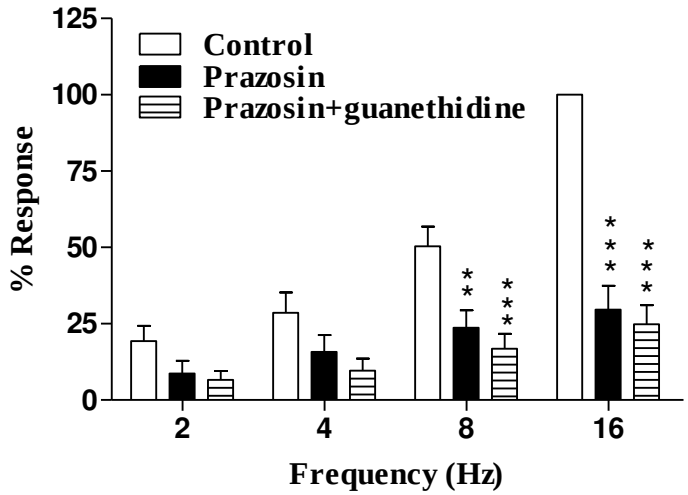



Fig. 3.2 Effects of prazosin (0.1 µM) alone and in combination with guanethidine (1 µM) (n=12), on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed under basal tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

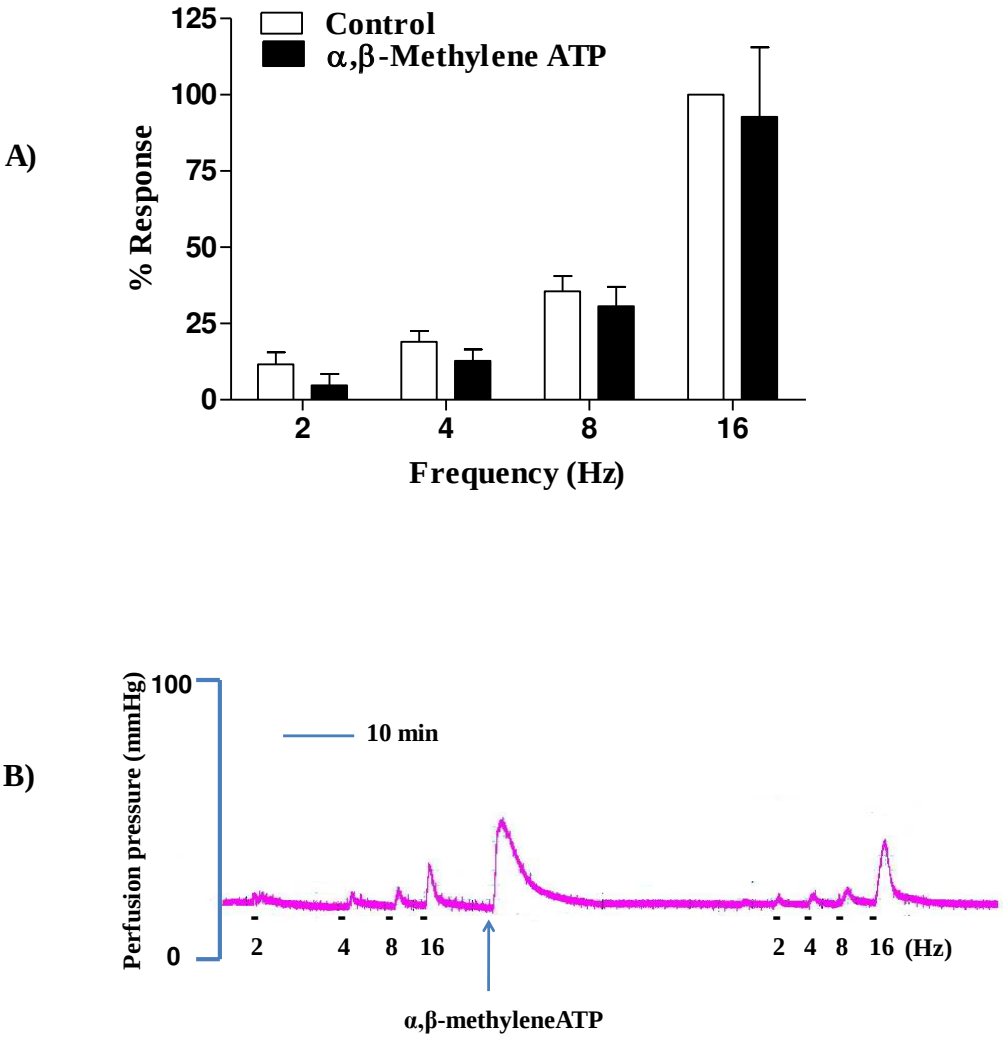



Fig. 3.3 A) Effect of α,β-methyleneATP (1 µM) (n=9) on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed under basal tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. B) Representative trace showing the effect of α,β-methyleneATP (1 µM) on vasoconstrictor responses to nerve stimulation in the porcine isolated mesenteric arterial bed under basal tone conditions.

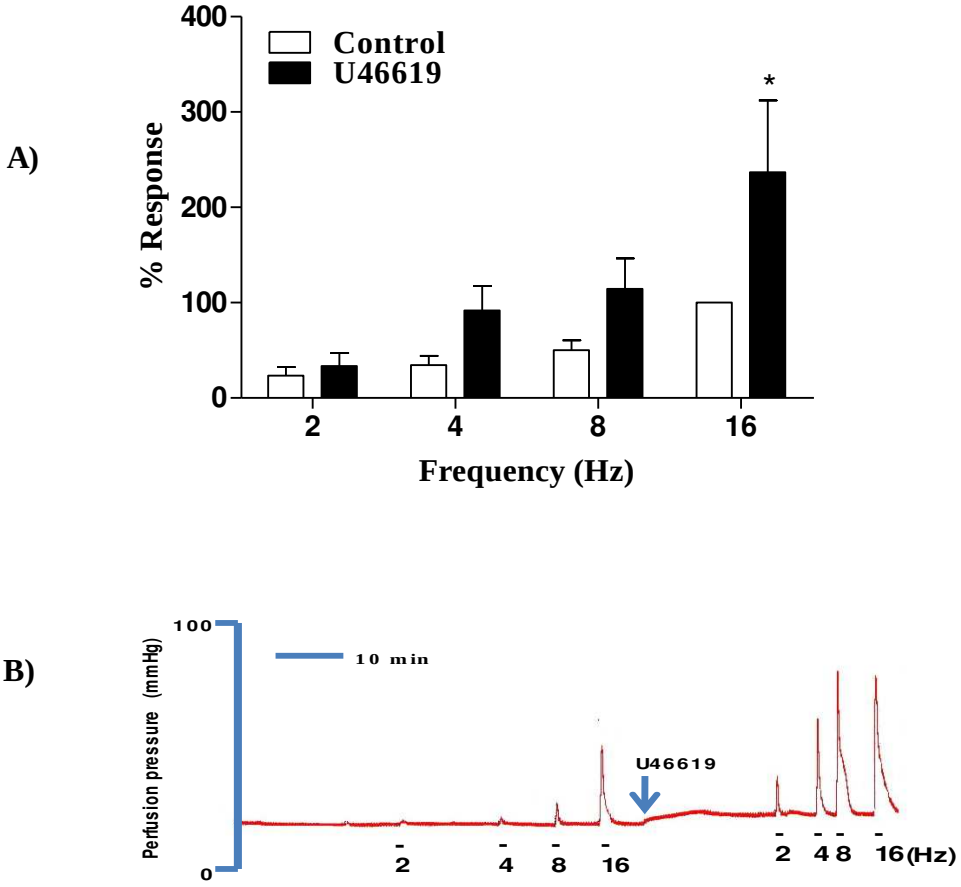



Fig. 3.4 A) Effects of U46619 (n=8), on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. * P < 0. 05 vs. control (ANOVA followed by Bonferroni post-hoc test). B) Representative trace showing effects of pre-constriction with U46619, on vasoconstrictor responses to nerve stimulation in the porcine isolated mesenteric arterial bed under basal and raised tone conditions.

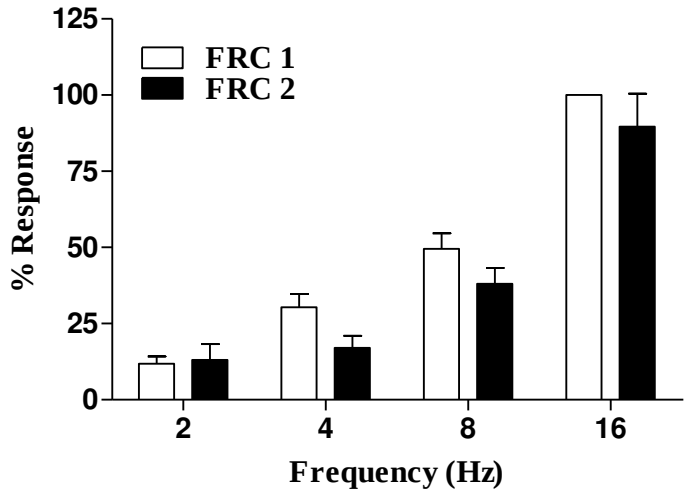



Fig. 3.5 Reproducibility of vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s). Open bars show the first frequency response curve (FRC 1) while closed bars show second frequency response curve (FRC 2) in the porcine isolated mesenteric arterial bed under raised tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve (n=5). Data are shown as mean ± standard error.

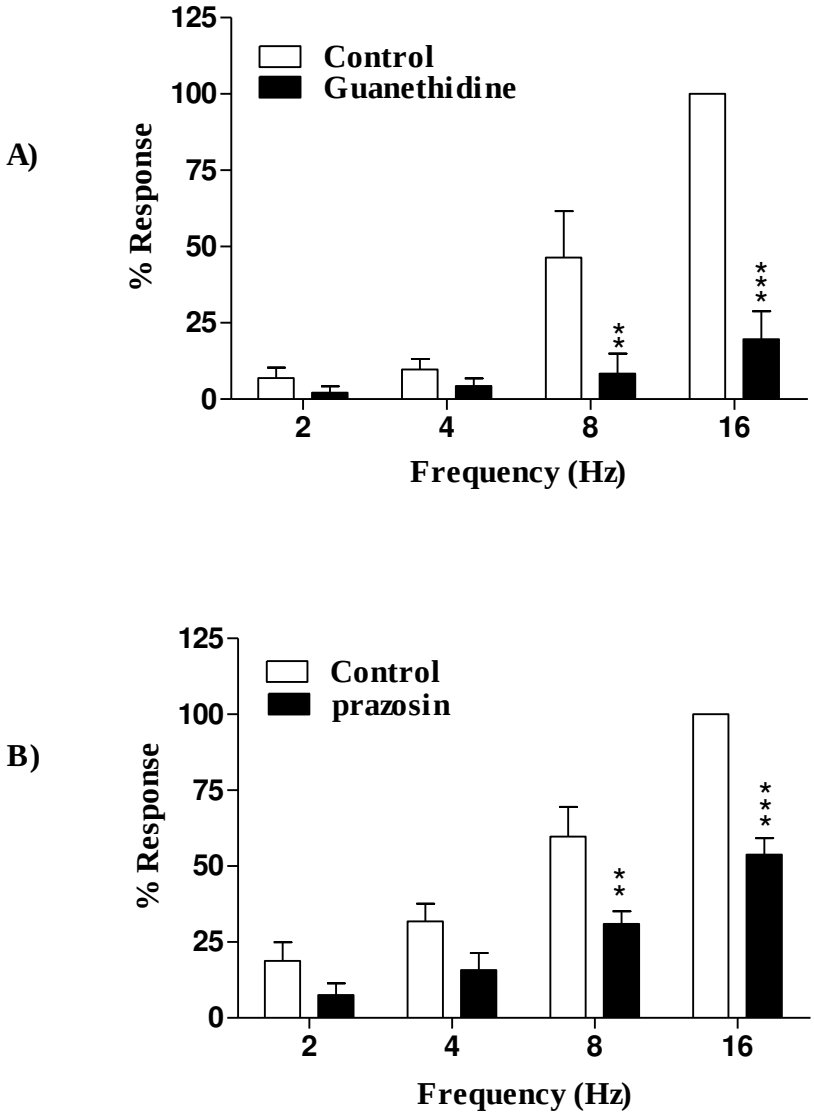
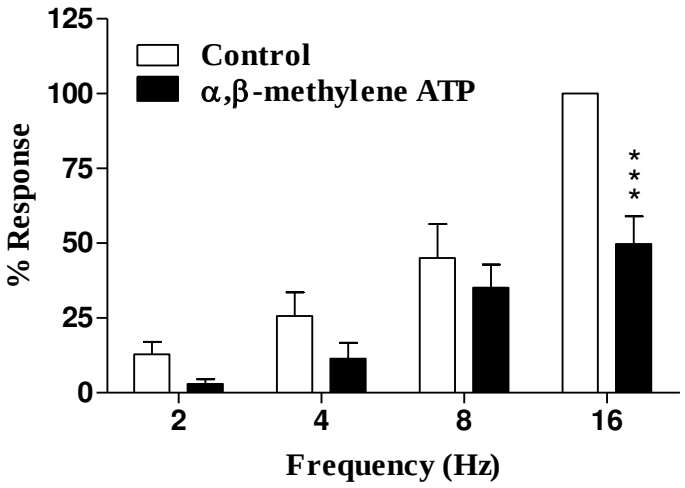



Fig. 3.6 Effects of A) guanethidine (1 µM) (n=4), or B) prazosin (0.1 µM) (n=12), on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed under raised tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

A)




B)

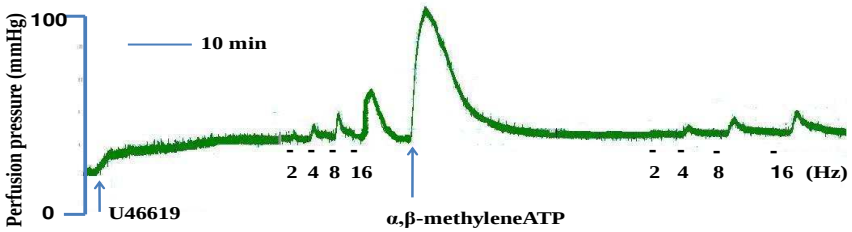



Fig. 3.7 A) Effects of α,β-methyleneATP (1 µM) (n=5), on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed under raised tone conditions induced by U46619 (10-25 nM). Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test). B) Representative trace showing effects of α,β-methyleneATP (1 µM) on vasoconstrictor responses to nerve stimulation in the porcine isolated mesenteric arterial bed under raised tone conditions.

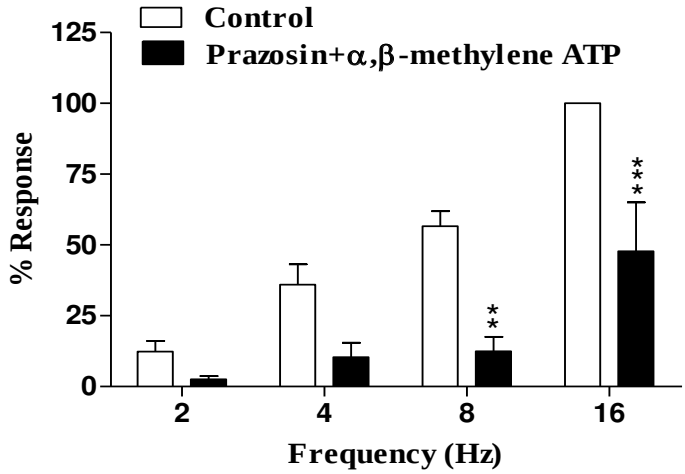



Fig. 3.8 Effects of prazosin (0.1 μM) plus α,β-methyleneATP (1 μM) (n=5) on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed under raised tone conditions. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error. ** $P < 0.01$ vs. control, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

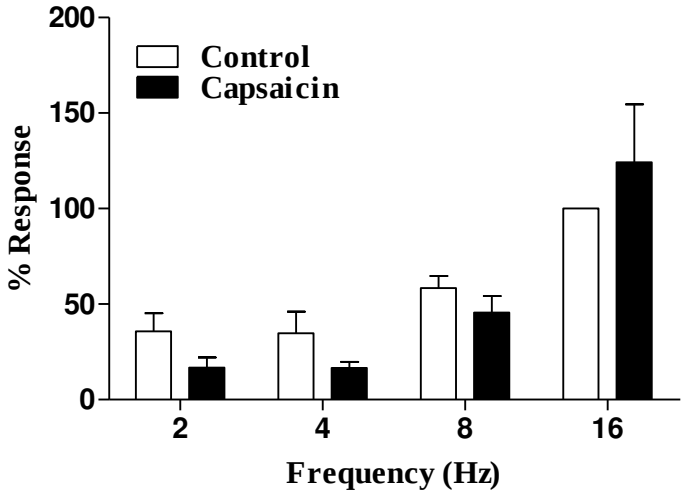



Fig. 3.9 Effect of capsaicin (1 µM) (n=9) on vasoconstrictor responses to electrical field stimulation (2-16 Hz, 1 ms, 90 V, 30 s) in the porcine isolated mesenteric arterial bed in the presence of U46619. Responses are expressed as a % of the control response to 16 Hz in the first frequency response curve. Data are shown as mean ± standard error.

## 3.5 DISCUSSION

The main finding of the present study is that pre-constriction with U46619 significantly enhanced the electrically-evoked vasoconstrictor responses in the porcine isolated mesenteric arterial bed, and this was associated with uncovering a role for ATP acting as a sympathetic co-transmitter, which was not evident under basal tone conditions.

Under basal tone conditions, the present results show that vasoconstrictor responses to nerve stimulation were sensitive to $\alpha_1$-adrenoceptor blockade by prazosin, but resistant to P2X receptor desensitization with α,β-methyleneATP. This indicates that the neuronally-evoked vasoconstrictor responses were mediated mainly via the stimulation of postjunctional $\alpha_1$-adrenoceptors. Since guanethidine produced a similar sized attenuation of the nerve-mediated response to that produced by prazosin, this suggests that NA is the principal sympathetic neurotransmitter acting in the porcine isolated mesenteric arterial bed under basal tone conditions. There was a response remaining at high frequencies that was not blocked by prazosin or guanethidine that, presumably, reflects direct activation of the smooth muscle with the stimulation parameters used. Since prazosin abolishes response to nerve stimulation in many blood vessels from several species such as the isolated perfused mesenteric vascular bed of rat (Eikenburg, 1984, Williams and Clarke, 1995), and pig prostatic small arteries (Recio et al., 2008), this has led to the widespread view that NA is the predominant neurotransmitter in the vasculature.

The failure of the P2X receptor desensitizing agent, α,β-methyleneATP to alter the neuronally-evoked vasoconstrictor responses under basal tone conditions in the porcine mesenteric arterial bed indicates that ATP is not involved as a functional sympathetic neurotransmitter in this bed. This observation is consistent with the results obtained from experiments in the rabbit perfused kidney and the rat mesenteric arterial bed under basal tone conditions where responses to EFS were blocked by prazosin but not by α,β-methyleneATP (von Kugelgen and Starke, 1985, Pakdeechote et al., 2007).

The resistance to α,β-methyleneATP under basal tone conditions in the present study was perhaps not surprising, since only a few studies have shown a role for ATP as a sympathetic neurotransmitter in blood vessels under basal tone. The studies include those on rabbit mesenteric arteries (von Kugelgen and Starke, 1985) and the rat perfused kidney (Schwartz and Malik, 1989). A possible explanation as to why in these studies ATP had an effect as a sympathetic neurotransmitter under basal tone conditions is that the involvement of ATP as a sympathetic neurotransmitter may differ among species and vary between vascular beds. Nevertheless, it is possible that in many blood vessels ATP is released from the sympathetic perivascular nerves under basal tone conditions but needs certain experimental conditions in order for it to be evident, which is consistent with an observation in human saphenous vein where ATP had no effect under basal tone conditions, although its release from nerves was demonstrated (Rump and von Kugelgen, 1994). Although a purinergic component was not observed at basal tone conditions in both porcine and rat mesenteric arterial beds (present study and Pakdeechote et

al., 2007), the absence of a purinergic component at basal tone does not appear to be characteristic of mesenteric arteries since in the rabbit mesenteric artery ATP is the principal transmitter (Ramme et al., 1987).

Pre-constriction with the thromboxane $A_2$ agonist, U46619, significantly increased the perfusion pressure and the size of vasoconstrictor responses to electrically-evoked nerve stimulation in the porcine isolated mesenteric arterial bed. This is in line with data obtained using the rat perfused mesenteric vascular bed (Pakdeechote et al., 2007), as well as in rabbit isolated portal vein (Stein and Trachte, 1989), rabbit isolated mesenteric artery (Trachte and Stein, 1989), and in the human saphenous vein, where U46619 enhanced vasoconstrictor responses to both EFS and exogenously applied NA (Vila et al., 2001). The present study is in line with these studies in suggesting a postjunctional mechanism by which U46619 enhanced the responses, since U46619 also enhanced the responses to exogenously applied α,β-methyleneATP in the porcine isolated mesenteric arterial bed. However because neurotransmitter release was not measured in this study, a presynaptic mechanism for U46619 cannot be excluded, although in the human saphenous vein U46619 did not alter electrically-evoked ($^3$H)-NA release unless it was used at concentrations higher than 1 µmol/ l (Molderings et al., 1994). In addition, the concentration-response curve to U46619 was not affected by prazosin in human saphenous vein suggesting that the action of U46619 does not involve release of NA (Vila et al., 2001).

After raising tone with U46619, prazosin attenuated the electrically-evoked vasocontractile responses, but was less effective than under basal tone conditions, particularly at higher frequencies. Thus there was a substantial prazosin-resistant response under raised tone conditions. Under raised tone conditions α,β-methyleneATP significantly blocked the response at higher frequencies, indicating that purinergic P2X receptors are involved in mediating part of the response. A clear involvement of ATP as a sympathetic co-transmitter has also been demonstrated in the rat mesenteric vascular bed after raising tone using endothelin or U46619 (Pakdeechote et al., 2007). It is unlikely that the contraction caused by α,β-methyleneATP have altered all contractions in general since electrically evoked responses were not altered by the addition of α,β-methyleneATP under basal tone conditions.

While it is possible that U46619 may increase the release of ATP from sympathetic nerves, a more likely explanation is a postjunctional mechanism. U46619 enhanced responses to the application of exogenous α,β-methyleneATP, showing the smooth muscle is more responsive to P2X receptor activation under these conditions. One possibility is that U46619 makes the membrane potential more positive such that there is an increase in the ATP-mediated opening of L-type $Ca^{2+}$ cannels leading to contraction. With a more negative membrane potential (in the absence of U46619), ATP via P2X receptors may not cause a sufficient depolarization to the open probability of these channels to promote a vasoconstriction. A similar process has been described in rat mesenteric arteries held under pressure; raising pressure from

30 to 90 mmHg resulted in depolarization and increased the function of ATP as a neurotransmitter (Rummery et al., 2007).

Under raised tone conditions the combination of prazosin and α,β-methyleneATP inhibited electrically-evoked responses at all frequencies but to a smaller extent than the sympathetic neuron blocker guanethidine, which essentially abolished the vasoconstrictor responses to EFS under these conditions. This raises possibility of the involvement of other neurotransmitters in mediating the vasoconstrictor response in the porcine arterial bed at raised tone. Neuropeptide Y (NPY) is a possible candidate. Although NPY is generally regarded as a neuromodulator, it has been shown to have a direct vasoconstrictor effect following release as a sympathetic co-transmitter in rat mesenteric arteries (Chu and Beilin, 1998, Han et al., 1998), and in human cerebral arteries (Edvinsson et al., 1994). Clearly the involvement of NPY as a sympathetic co-transmitter needs to be investigated further in the porcine isolated mesenteric arterial bed.

It has been shown that EFS of capsaicin-sensitive sensory nerves elicited vasorelaxation mediated by calcitonin gene-related peptide (CGRP) in the rat isolated mesenteric vascular bed (Kawasaki et al., 1988, Rubino et al., 1992). Furthermore it has been shown that depletion of capsaicin-sensitive sensory afferent nerves of their neurotransmitters by capsaicin enhanced the vasocontractile response to EFS in the rat isolated mesenteric vascular bed (Pakdeechote et al., 2007). However, in the present study, pre-treatment with capsaicin did not alter electrically-evoked vasocontractile responses in the

porcine isolated mesenteric arterial bed. This suggests that sensory nerves do not play a significant role in this preparation.

In conclusion the present study has shown that, at basal tone, NA appears to be the only functional (contractile) sympathetic neurotransmitter. In contrast, at raised tone there is an additional involvement of ATP as a sympathetic co-transmitter with NA. Raising the tone with U46619 also revealed the possibility that another neurotransmitter, in addition to NA and ATP, might be involved in mediating vascular smooth muscle sympathetic neurogenic contraction.

# CHAPTER 4

# SYMPATHETIC NEUROTRANSMISSION IN PORCINE ISOLATED ARTERIES

## 4.1 INTRODUCTION

It is well established that sympathetic perivascular nerves co-store and co-release not only NA but also ATP, and NPY (Lundberg et al., 1983, Ekblad et al., 1984, Burnstock, 1988). There is, however, considerable variation in their relative proportion between vessels and species. In most blood vessels NA is the main neurotransmitter acting through postjunctional $\alpha_1$-adrenoceptors (Angus et al., 1988) and occasionally $\alpha_2$-adrenoceptors (Flavahan et al., 1987, Dunn et al., 1989), while NPY and ATP are co-transmitters. NPY acts mainly through $Y_1$ receptors as a neuromodulator, with some evidence of a direct postjunctional effect in some preparations. For example, 30% of the vasoconstrictor response produced by sympathetic nerve stimulation in the rat mesenteric vascular bed was mediated by NPY (Han et al., 1998). Nevertheless there is a consensus that NPY has a more important role as a neuromodulator in sympathetic vascular reflexes, potentiating the vasoconstrictor effects of NA, for example, in rabbit femoral arteries and cerebral arteries of cat (Edvinsson et al., 1984, Edvinsson, 1985), or facilitating the effect of ATP, for example, in human saphenous vein (Donoso et al., 2004). ATP mediates its effects through P2X (ion channel) and P2Y (G protein-coupled) receptors (Erlinge and Burnstock, 2008). $P2X_1$ receptors are most widely distributed in vascular smooth muscle and mediate the effects of ATP as a sympathetic co-transmitter (Lewis and Evans, 2000, Wang et al., 2002). The importance and

the degree of involvement of ATP in sympathetic neurotransmission varies, depending on species, vascular bed, size of blood vessel and experimental conditions. For example, it has been demonstrated that the role of ATP as a sympathetic neurotransmitter increases as the size of the blood vessel decreases (Gitterman and Evans, 2001). Indeed, in the submucosal arterioles of the guinea-pig, ATP was the sole sympathetic neurotransmitter mediating contractile responses to sympathetic stimulation, with NA acting as a neuromodulator (Evans and Surprenant, 1992). Another important factor which has an effect on the relative contribution of ATP as a sympathetic neurotransmitter is the experimental conditions used. For example, raising the pressure from 30 to 90 mmHg revealed that, at the higher pressure, ATP was the predominant sympathetic neurotransmitter in rat pressurised mesenteric arteries (Rummery et al., 2007). Another study has shown that raising the tone with U46619, a thromboxane $A_2$ agonist, revealed a functional role for ATP as a sympathetic co-transmitter in the rat perfused mesenteric vascular bed, where no significant role was observed at basal tone conditions (Pakdeechote et al., 2007).

In chapter 3 I showed that ATP was involved in the mediation of electrically-evoked contractions in the porcine isolated mesenteric arterial bed but only under raised tone conditions. Therefore, in the present study, the aim was, to investigate the role of ATP in different sized vessels from this vascular tree, specifically first and third order arteries. The possible involvement of other neurotransmitters in the mediation of electrically-evoked contractile responses under basal and raised tone conditions, and the involvement of postjunctional

mechanism in the enhanced responses in the presence of raised tone conditions were also investigated.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Tissue preparation

Porcine mesenteries were obtained from a local abattoir and transported to the laboratory in buffer placed on ice. First and third order porcine mesenteric arteries were set up for isometric recording as described in section 2.2.

### 4.2.2 Characterization of electrically-evoked contractile responses in porcine mesenteric first order arteries under basal tone conditions

Under basal tone conditions, two frequency response curves (FRCs) were generated separated by a 30 min interval. These experiments were used as time controls. In all experiments a FRC was generated and acted as an internal control after which the tissue was exposed to either tetrodotoxin (TTX) (1 μM), a sodium channel blocker (Lombard et al., 1982), the sympathetic neurone blocker guanethidine (1 μM), prazosin (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, RX 811059 (1 μM), an $\alpha_2$-adrenoceptor antagonist (Berridge and Roach, 1986), α,β-methyleneATP (1 μM), a P2X receptor desensitising agent, or capsaicin (1 μM), a TRPV1 receptor agonist (Caterina and Julius, 2001) to investigate their effects on the contractile response obtained to EFS (2-32 Hz, 1 ms, 90 V, 30 s). All antagonists were incubated for 30 min before testing their effects on the electrically-evoked contractile responses.

### 4.2.3 Effects of capsaicin treatment on electrically-evoked contractile responses in porcine mesenteric first order arteries under basal tone conditions

After generating a FRC, capsaicin (1 μM, for 30 min) was used to deplete the sensory nerves of their neurotransmitters. The tissue was then washed with Krebs-Henseleit buffer for 30 min, and a second FRC was obtained. In other experiments the same protocol was adopted, but ethanol (0.1%) was used as a vehicle control.

### 4.2.4 Characterization of electrically-evoked contractile responses in porcine mesenteric first order arteries under raised tone conditions

In each experiment, prior to exposing the tissue to EFS, U46619 (5-25 nM), a thromboxane $A_2$ agonist, was used to raise the tone to about 25% of the level of the contraction obtained during the second KCl response, since preliminary experiments showed that preconstriction to more than 40% may lead to limitation of the smooth muscle ability to further contract. In some preparations a FRC was obtained under basal tone conditions before U46619 was added to raise the tone and a second FRC was conducted under raised tone conditions. In other preparations, after raising the tone, three FRCs were generated without the addition of antagonists; these were used as time controls. In related experiments, after raising the tone, a FRC was generated and acted as an internal control and the raised tone was maintained at the same level by the further addition of U46619, if required. After generating the first FRC, vessels were exposed to either guanethidine (1 μM), prazosin (0.1 μM), RX

811059 (1 μM), α,β-methyleneATP (1 μM), or BIBP3226 (1 μM) (Rudolf et al., 1994). In other experiments the tissue was exposed to the sequential effects of prazosin (0.1 μM) followed by the combination of prazosin (0.1 μM) plus either α,β-methyleneATP (1 μM), RX 811059 (1 μM) or BIBP3226 (1 μM). Alternatively, vessels were exposed to RX 811059 (1 μM) followed by the combination of RX 811059 (1 μM) plus α,β-methyleneATP (1 μM). All antagonists were incubated for 30 min before electrically-evoked contractile responses were obtained.

### 4.2.5 Characterization of electrically-evoked contractile responses in porcine mesenteric third order arteries under basal tone conditions

Three frequency response curves were generated, with a 30 min interval between them, in the absence of antagonist, as controls. In each experiment, a FRC was produced and acted as an internal control after which the tissue was exposed to the sequential effects of prazosin (0.1 μM) followed by prazosin (0.1 μM) plus guanethidine (1 μM), or α,β-methyleneATP (1 μM) alone. All antagonists were incubated for 30 min before testing their effects on the electrically-evoked contractile responses.

### 4.2.6 Characterization of electrically-evoked contractile responses in porcine mesenteric third order arteries under raised tone conditions

In each experiment prior to exposing the tissue to EFS, U46619 (5-10 nM) was used to raise the tone to a level of about 25% of the contraction in response to the second KCl response. In some preparations a FRC was obtained under

basal tone conditions then U46619 was added to raise the tone and a second FRC was conducted. In all other experiments, after raising tone, a FRC was generated and acted as an internal control after which the tissue was exposed to the sequential application of prazosin (0.1 μM) followed either by the combination of prazosin (0.1 μM) plus guanethidine (1 μM), or prazosin (0.1 μM) plus α,β-methyleneATP (1 μM), to investigate their effect on the contractile response obtained to EFS (2-32 Hz, 1 ms, 90 V, 30 s). The raised tone was maintained at the same level by the addition of U46619 if required between FRCs. All antagonists were incubated for 30 min before testing their effects on the electrically-evoked contractile responses.

### 4.2.7 Responses to α,β-methyleneATP in porcine mesenteric first order arteries under basal tone conditions

After a 60 min equilibration period, responses to a single application of α,β-methyleneATP (1 μM) under basal tone conditions were conducted. The tissue was then washed twice with Krebs-Henseleit buffer for 15 min before a second application of α,β-methyleneATP (1 μM). In some experiments tissues were exposed to the L-type calcium-sensitive channel nifedipine (1 μM) (Surprenant et al., 1983) for 30 min before the second response to α,β-methyleneATP (1 μM).

### 4.2.8 Responses to α,β-methyleneATP in porcine mesenteric first order arteries under raised tone conditions

Responses to a single application of α,β-methyleneATP (1 μM) were obtained under basal tone conditions. The tissue was then washed twice with Krebs-Henseleit buffer. After 30 min U46619, a thromboxane $A_2$ agonist (5-25 nM) was then added to pre-constrict the vessel to about 25% of the second KCl response. When a stable contraction to U46619 was achieved, a second response to α,β-methyleneATP (1 μM) was obtained. In separate experiments the same protocol was conducted but endothelin-1 (1-2 nM), a vasoconstrictor agent was used instead.

In some experiments, before exposing the tissue to a second application of α,β-methyleneATP (1 μM), nifedipine (1 μM) was applied for 30 min beforehand. In experiments where nifedipine was used, the vessels were pre-constricted to 35%-45% of the second KCl response. Thus when nifedipine was added in these vessels decreased back to 25% of the second KCl response. Only after reaching a stable level of pre-constriction was a second response to α,β-methyleneATP (1 μM) obtained.

### 4.2.9 Responses to exogenous noradrenaline in porcine mesenteric first order arteries under basal and raised tone conditions

Preliminary experiments showed an element of desensitization when concentration response curves were obtained to exogenous NA. Thus to try to avoid desensitization, a single application of NA (1 μM or 0.3 μM) was used. In some experiments responses to NA (1 μM or 0.3 μM) were obtained under

basal tone conditions. The tissue was washed twice with Krebs-Henseleit buffer for 30 min and U46619 (5-25) nM was then added to pre-constrict the vessel to about 25% of the second KCl response. When a stable contraction to U46619 was achieved, a second response to NA (1 µM or 0.3 µM) was obtained.

## 4.3 STATISTICAL ANALYSIS

Results are expressed as the mean ± S.E.M. Statistical comparisons were made by two way analysis of variance (ANOVA) with Bonferroni post-hoc test or Student's paired or unpaired t-test if the data were normally distributed (checked by Shapiro-Wilk normality test) while Mann-Whitney test was used if the data were not normally distributed. A value of $P < 0.05$ was taken to indicate statistical significance.

## 4.4 RESULTS

### 4.4.1 Effects of prazosin, α,β-methyleneATP and RX811059 on responses to EFS in porcine mesenteric first order arteries under basal tone conditions

Under basal tone conditions EFS produced contractile responses which were frequency-dependent, and reproducible (n=5) (Fig. 4.1). Tetrodotoxin (TTX) (1 µM), a sodium channel blocker, almost completely abolished the response to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 4.2A). Similarly guanethidine (1 µM), a sympathetic neuron blocker, almost

abolished the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=10) (Fig. 4.2B).

Prazosin (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 4.3A) to a similar extent to that produced by TTX or guanethidine (see Fig. 4.2A and B). α,β-methyleneATP (1 μM), a P2X receptor desensitizing agent, caused a transient contraction (1.7 ± 0.1 g, n=8) which returned to the baseline before the construction of the next FRC, but had no significant effect on the contractile responses to EFS in porcine mesenteric first order arteries (n=8) (Fig. 4.3B).

When assessed using ANOVA, RX811059, an $\alpha_2$-adrenoceptor antagonist (1 μM), was shown to significantly attenuate the contractile responses to EFS (Fig. 4.3C). Although the two FRCs were significantly different ($P < 0.05$, ANOVA) (n=7) there was no significant change at any single frequency upon post-hoc analysis. The effect of RX811059 seemed greater at lower frequencies than at higher frequencies.

### 4.4.2 Effect of capsaicin on electrically-evoked contractile responses in porcine mesenteric first order arteries under basal tone conditions

Capsaicin (1 μM), a TRPV1 receptor agonist, significantly increased the electrically-evoked contractile responses ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=10) (Fig. 4.4A). Similarly ethanol increased the electrically-evoked contractile response ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=8) (Fig. 4.4B) although none of the individual

frequencies were statistically significantly different. Capsaicin and ethanol both increased responses to EFS. However, the effect of ethanol was just as large as that of capsaicin.

### 4.4.3 Effects of prazosin, α,β-methyleneATP, RX811059 and BIBP3226 on responses to EFS in porcine mesenteric first order arteries under raised tone conditions

U46619 (5-25 nM) contracted the first order mesenteric arteries by 20 ± 4% of the KCl response (n=14). Under raised tone conditions, EFS produced contractile responses which were frequency-dependent and slightly larger than under basal tone conditions ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=14) (Fig. 4.5). Under raised tone conditions three consecutive FRCs were reproducible (n=5) (Fig. 4.6).

Guanethidine (1 μM), almost abolished the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=4) (Fig. 4.7A). Prazosin (0.1 μM), significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 4.7B) but there was a substantial prazosin-resistant component under raised tone conditions.

α,β-methyleneATP (1 μM), a P2X receptor desensitizing agent, caused a transient contraction (4.6 ± 0.6 g, n=12), which returned to the same level of tone before the construction of the next FRC. When assessed using ANOVA, α,β-methyleneATP was shown to significantly attenuate the contractile responses to EFS. Although the two FRCs were significantly different ($P <$

0.05, ANOVA) (n=12) (Fig. 4.7C) there was no significant change at any single frequency upon post-hoc analysis. The addition of α,β-methyleneATP (1 μM) after prazosin further inhibited the residual pressor response at all frequencies reaching significance at 32 Hz ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=10) (Fig. 4.9A). Thus the combined effect of α,β-methyleneATP and prazosin was more evident than that produced by prazosin alone.

RX811059 (1 μM), an $\alpha_2$-adrenoceptor antagonist, significantly attenuated the contractile responses to EFS. Although the two FRCs were significantly different there was no significant difference at any individual frequency ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 4.8A). These results are similar to those produced by RX811059 under basal tone conditions (see Fig. 4.3C). Further addition of RX811059 after prazosin produced a trend of a further inhibitory effect but this was not significant (n=4) (Fig. 4.9B).

The addition of α,β-methyleneATP (1 μM) after RX811059 (1 μM) inhibited the residual response left after RX811059 showing that the combined effect of α,β-methyleneATP and RX811059 had more inhibitory effect than that produced by the addition of either of the drugs alone on the electrically-evoked contractile responses in porcine mesenteric first order arteries under raised tone conditions ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 4.10).

BIBP3226 (1 μM), an neuropeptide Y1 receptor antagonist, alone had no significant effect on the electrically-evoked contractile responses at raised tone (n=5) (Fig. 4.8B). The addition of BIBP3226 (1 μM) after prazosin had no significant effect on the electrically-evoked contractile responses in porcine mesenteric first order arteries (n=7) (Fig. 4.9C).

### 4.4.4 Effects of prazosin and α,β-methyleneATP on responses to EFS in porcine mesenteric third order arteries under basal tone conditions

In porcine mesenteric third order arteries, under basal tone conditions EFS produced contractile responses which were frequency-dependent although large responses were only seen at high frequencies. Under these conditions three consecutive FRCs were reproducible (n=5) (Fig. 4.11). Prazosin (0.1 μM) significantly inhibited the contractile responses to EFS at basal tone ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 4.12A). Further addition of guanethidine (1 μM) reduced the residual response after prazosin but this further reduction was not significantly different from that produced by prazosin alone (n=6) (Fig. 4.12A).

α,β-methyleneATP (1 μM) caused a transient contraction (0.8 ± 0.2 g, n=8) which returned to baseline before the construction of the next FRC, and did not alter the contractile responses under basal tone conditions (Fig. 4.12B) (n=6).

### 4.4.5 Effects of prazosin and α,β-methyleneATP on responses to EFS in porcine third order mesenteric arteries under raised tone conditions

U46619 (5-10 nM), contracted the third order arteries by 20 ± 4% of KCl response (n=14). Under these conditions EFS produced contractile responses which were frequency-dependent and were modestly larger than under basal tone conditions particularly at lower frequencies. Although the two FRCs were significantly different none of the responses individual frequencies were significantly different upon post-hoc analysis ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 4.13).

Prazosin (0.1 µM) significantly inhibited the contractile responses to EFS under raised tone conditions ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 4.14A). Subsequent addition of guanethidine (1 µM) reduced the residual response further, at both 16 and 32 Hz ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=6) (Fig. 4.14A).

α,β-methyleneATP (1 µM) caused a transient contraction (1.8 ± 0.2 g, n=9) which returned to the baseline before beginning the next FRC. This contraction was significantly larger than that produced by α,β-methyleneATP (1 µM) under basal conditions ($P < 0.01$, Student's unpaired t-test, n=7). α,β-methyleneATP attenuated the contractile response to EFS, at all frequencies, reaching statistical significance at 32 Hz ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 4.14B) in porcine mesenteric third order arteries under raised tone conditions.

### 4.4.6 Effects of nifedipine on responses to α,β-methyleneATP under basal conditions in porcine first order mesenteric arteries

Under conditions of basal tone α,β-methyleneATP (1 μM), produced a contractile response that was larger upon the second exposure ($P < 0.05$, Student's paired t-test) (n=8) (Fig. 4.15). Nifedipine (1 μM) had no significant effect on the response to α,β-methyleneATP (1 μM), (n=8) (Fig. 4.16A and B representative trace).

### 4.4.7 Effects of nifedipine on responses to α,β-methyleneATP under raised tone conditions in porcine mesenteric first order arteries

In the presence of U46619 (5-25 nM), the response to α,β-methyleneATP (1 μM) was significantly enhanced ($P < 0.001$, Student's paired t-test) (n=8) (Fig. 4.17A and B representative trace). In the presence of U46619, nifedipine (1 μM) attenuated the response to α,β-methyleneATP (1 μM) ($P < 0.01$, Student's unpaired t-test) (n=8) (Fig. 4.18).

### 4.4.8 Effects endothelin-1 on responses to α,β-methyleneATP in porcine mesenteric arteries

In the rat perfused mesenteric vascular bed, pre-treatment with endothelin-1, a vasoconstrictor agent, enhanced electrically-evoked vasoconstrictor responses (Pakdeechote et al., 2007). In the present study we investigated the effect of endothelin-1 on responses to α,β-methyleneATP (1 μM) in the porcine mesenteric arteries.. Endothelin-1 (1-2 nM) contracted porcine mesenteric arteries and significantly enhanced the response to α,β-methyleneATP (1 μM) ($P < 0.05$, Student's paired t-test) (n=9) (Fig. 4.19).

### 4.4.9 Effects of U46619 on exogenous NA in porcine first order mesenteric arteries

NA (1 µM) produced a contractile response which was enhanced in the presence of U46619 ($P < 0.01$, Student's paired t-test) (n=8) (Fig. 4.20A and B representative trace). However, the enhancement produced by NA (1 µM), was modest thus, a lower concentration of NA (0.3 µM) was used to avoid any underestimation of a possible enhancement that may occur when a higher concentration is used. NA (0.3 µM) produced a contractile response which was enhanced in the presence of U46619 ($P < 0.01$, Student's paired t-test) (n=8) (Fig. 4.21A and B representative trace).

### 4.4.10 Comparison between the effects of α,β-methyleneATP on electrically-evoked contractile responses in porcine mesenteric first and third order arteries

α,β-methyleneATP (1 µM), inhibited the electrically-evoked contractile responses in both first and third order arteries at 32 Hz under raised tone conditions. It seems that the percentage of inhibition produced by α,β-methyleneATP (1 µM) greater in third order arteries than that produced in first order arteries. However that was statistically not significantly (n=9) (Fig. 4.22).

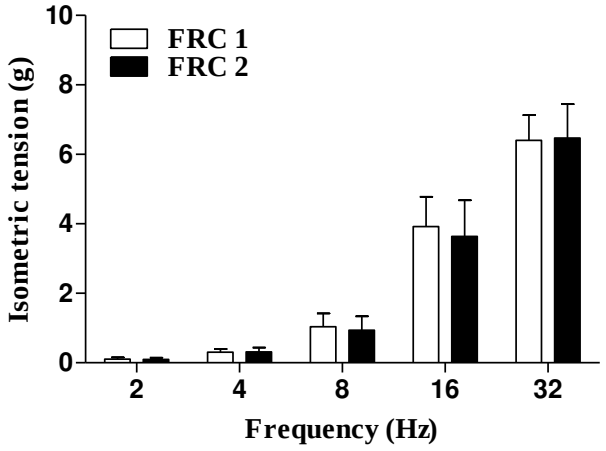



Fig. 4.1 Reproducibility of contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V). Open bars show the first frequency response curve (FRC 1) while closed bars show second frequency response curve (FRC 2) (n=5) in porcine mesenteric first order arteries under basal tone conditions. Each bar represents mean ± standard error.

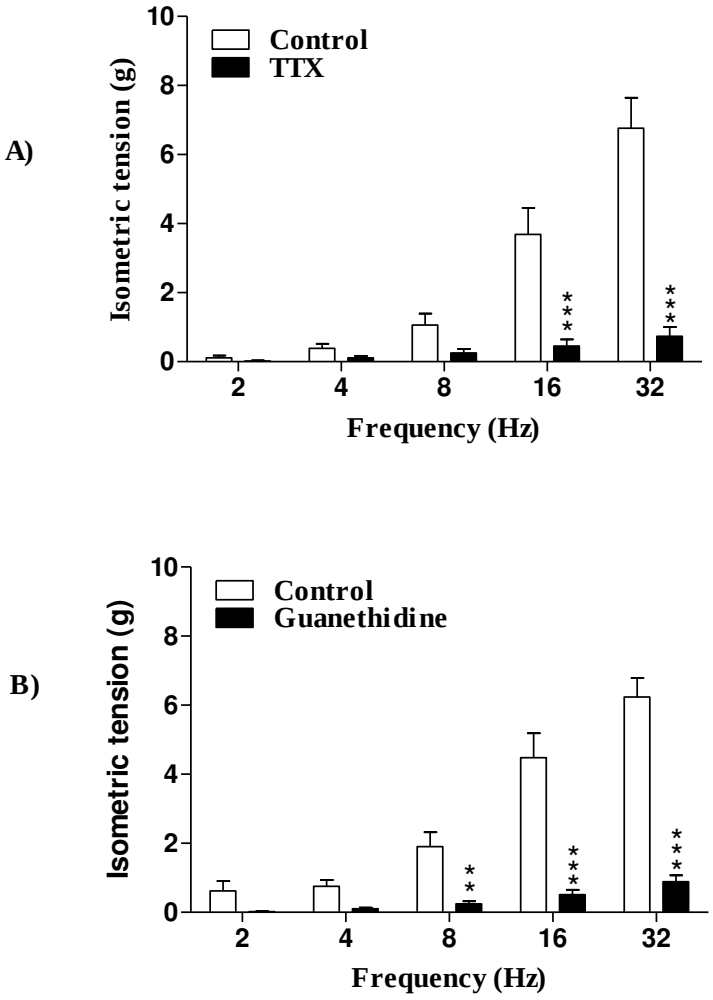
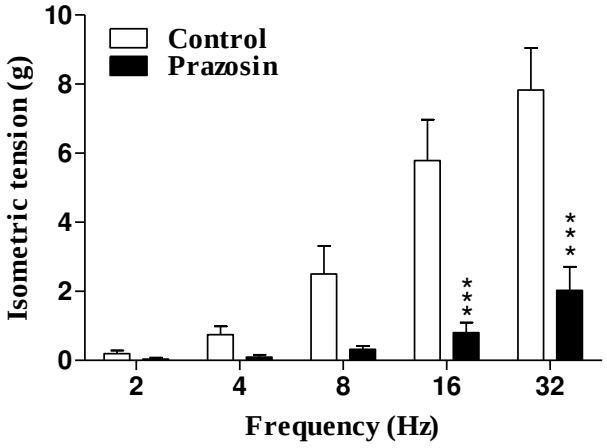
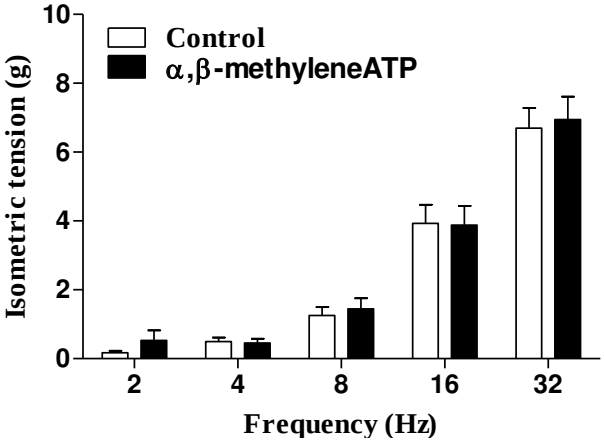



Fig. 4.2 Effects of A) tetrodotoxin (TTX, 1 μM) (n=5), or B) guanethidine (1 μM) (n=10) on contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30s, 90 V) in porcine mesenteric first order arteries under basal tone conditions. Each bar represents mean ± standard error. ** $P < 0.01$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

A)




B)




C)

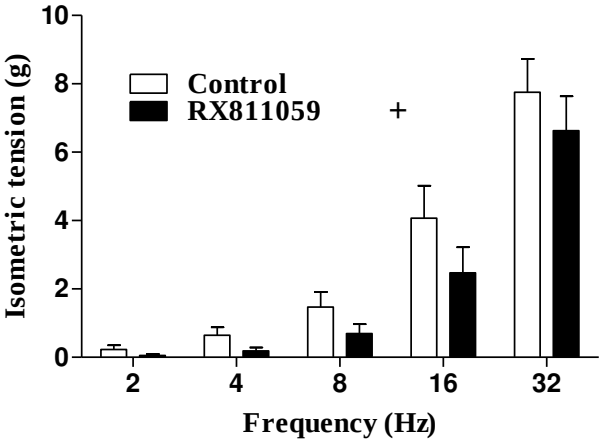
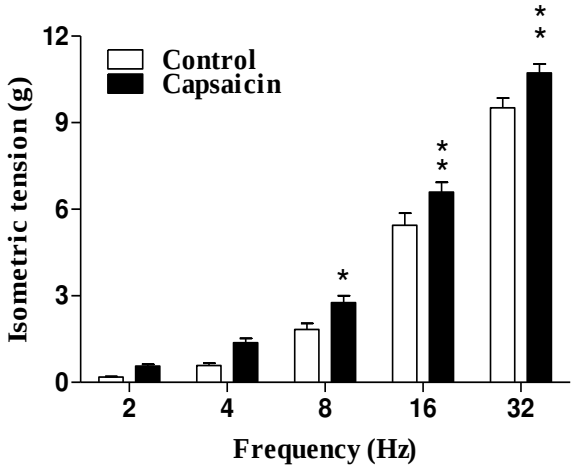



Fig. 4.3 Effects of A) prazosin (0.1 μM) (n=5), B) α,β-methyleneATP (1 μM) (n=8), or C) RX811059 (1 μM) (n=7) on contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30s, 90 V) in porcine mesenteric first order arteries under basal tone conditions. Each bar represents mean ± standard error. *** $P < 0.001$ vs. control. + shows a significant difference between curves ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test).

A)




B)

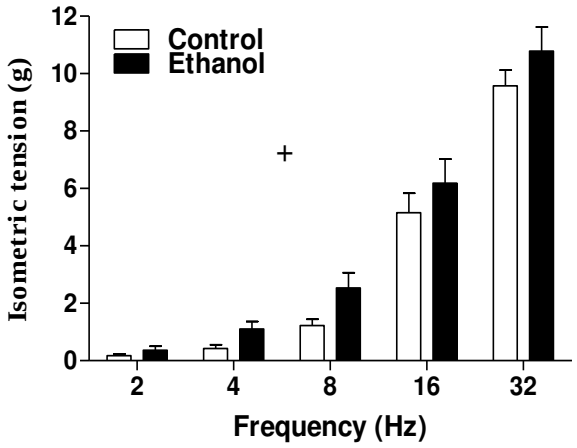



Fig. 4.4 Effects of A) capsaicin (1 μM) (n=10), or B) ethanol (0.1%) (n=8), on contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30s, 90 V) in porcine mesenteric first order arteries under basal tone conditions. Each bar represents mean ± standard error. * $P < 0.05$, ** $P < 0.01$ vs. control. + shows a significant difference between curves ($P < 0.05$, ANOV followed by Bonferroni post-hoc test).

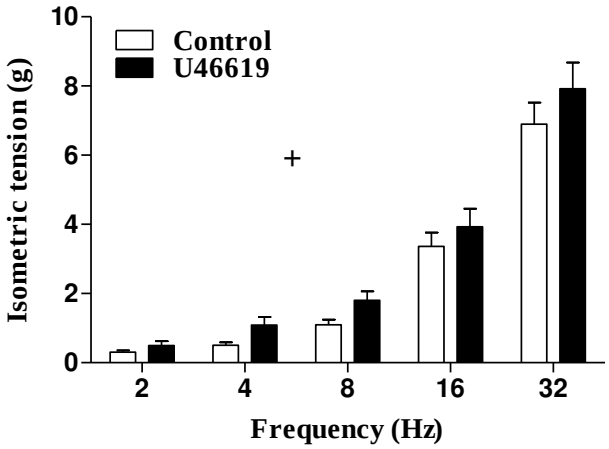



Fig. 4.5 Contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30s, 90 V) under basal tone conditions (control), and in the presence of U46619 (n=14) in porcine mesenteric first order arteries. Each bar represents mean ± standard error. + shows a significant difference between curves ($P < 0.05$, ANOV followed by Bonferroni post-hoc test).

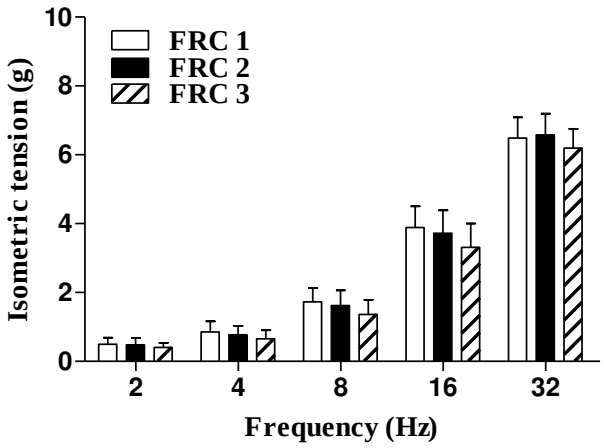



Fig. 4.6 Reproducibility of contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V). Open bars show the first frequency response curve (FRC 1), while closed bars show the second frequency response curve (FRC 2) and striped bars show the third frequency response curve (FRC 3) (n=5) in porcine mesenteric first order arteries under raised tone conditions. Each bar represents mean ± standard error.

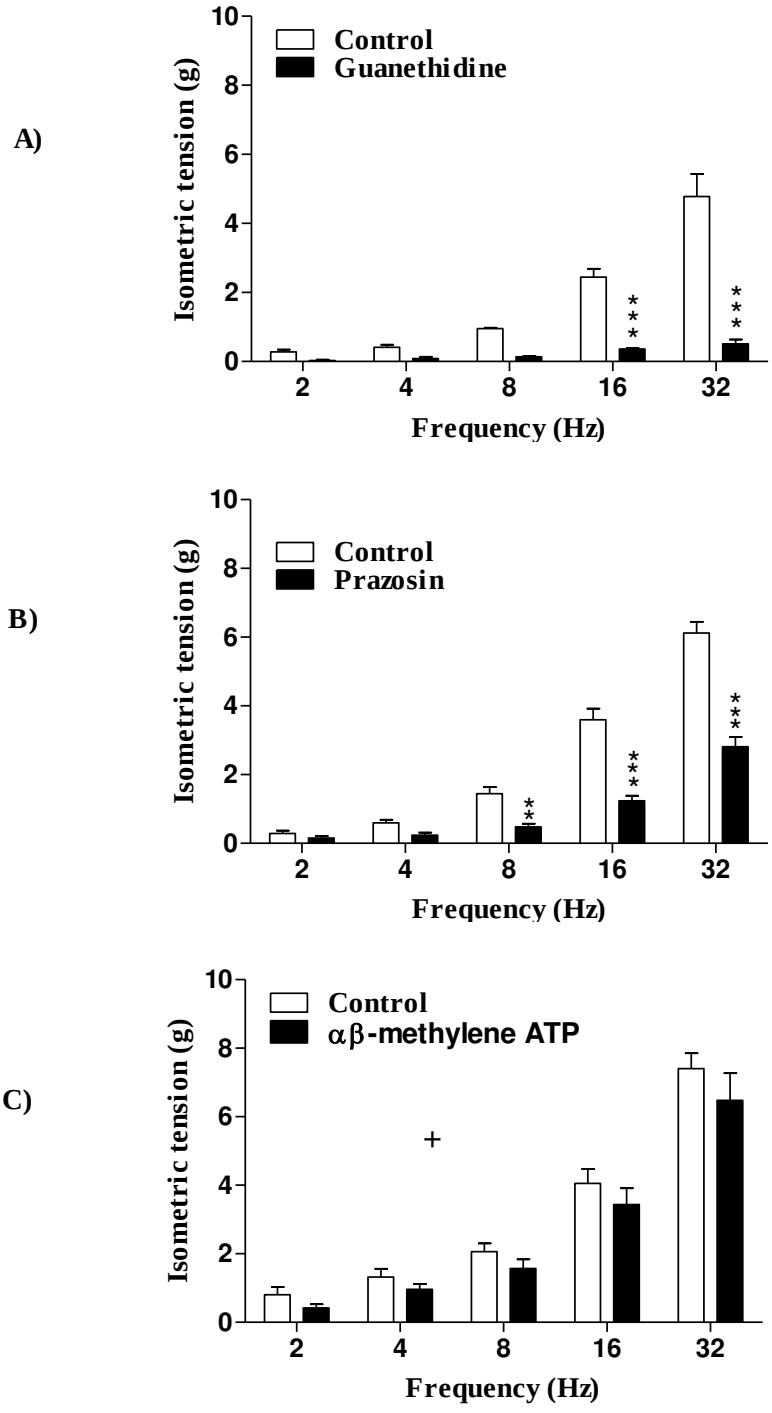



Fig. 4.7 Effects of A) guanethidine (1 μM) (n=4), B) prazosin (0.1 μM) (n=12), or C) α,β-methyleneATP (1 μM) (n=12), on contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V) in porcine mesenteric first order arteries under raised tone conditions. Each bar represents mean ± standard error. ** P < 0.01, *** P < 0.001 vs. control. + shows a significant difference between curves (P < 0.05, ANOVA followed by Bonferroni post-hoc test).

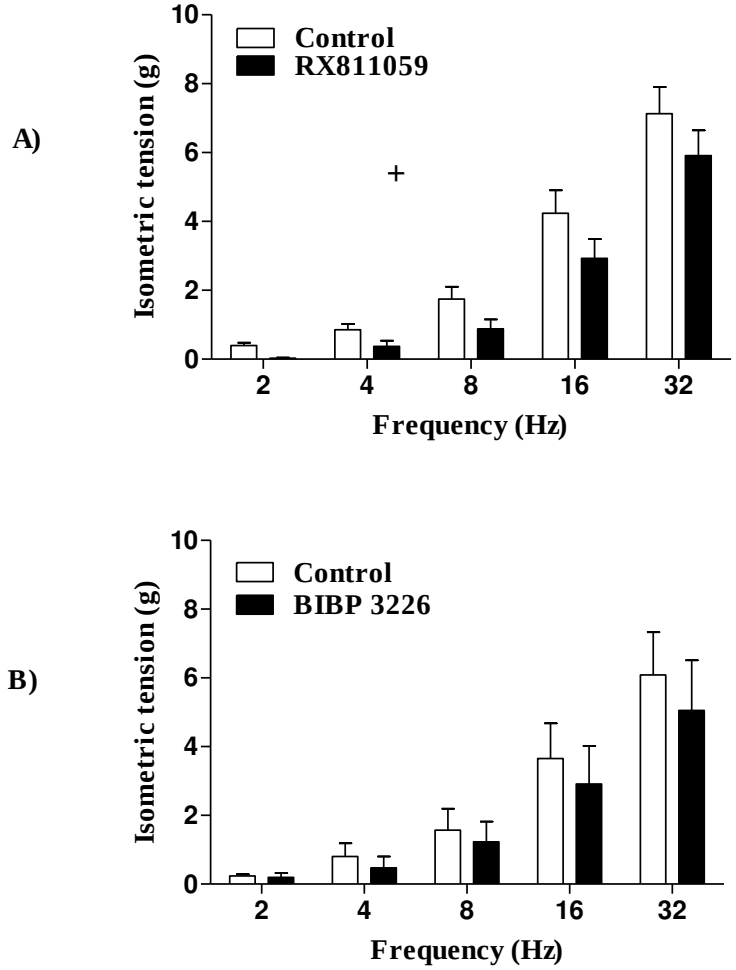



Fig. 4.8 Effects of A) RX811059 (1 μM) (n=12), or B) BIBP3226 (1 μM) (n=5), on contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V) in porcine mesenteric first order arteries under raised tone conditions. Each bar represents mean ± standard error. + shows a significant difference between curves ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test)

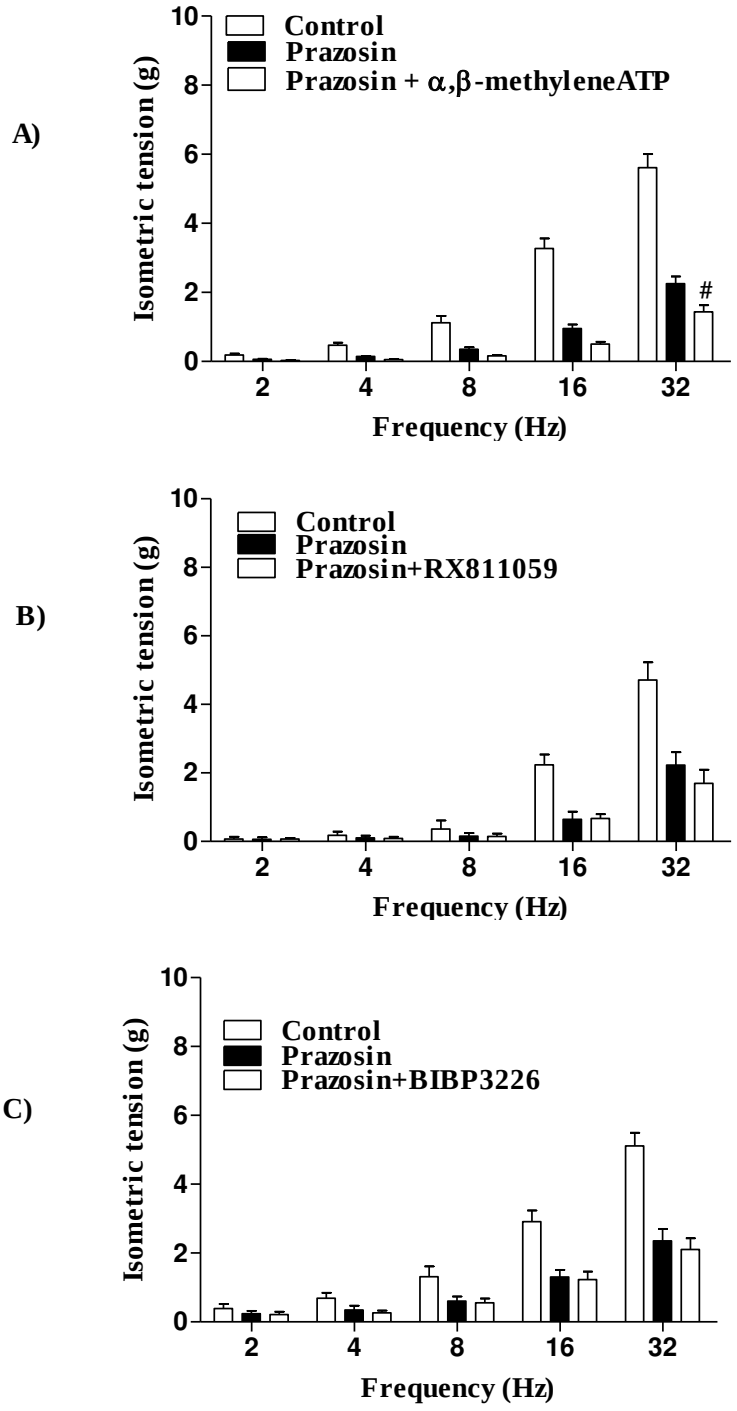



Fig. 4.9 Effects of A) the sequential addition of prazosin (0.1 μM), and α,β-methyleneATP (1 μM) (n=10), B) of prazosin (0.1 μM) and RX811059 (1 μM) (n=4), or C) of prazosin (0.1 μM) and BIBP3226 (1 μM) (n=7), on responses to electrical field stimulation (2-32 Hz, 1 ms, 30 s, 90 V) in porcine mesenteric first order arteries under raised tone conditions. Each bar represents mean ± standard error. Responses after prazosin were significantly inhibited in all sets of experiments. # $P < 0.001$ vs. prazosin (ANOVA followed by Bonferroni post-hoc test).

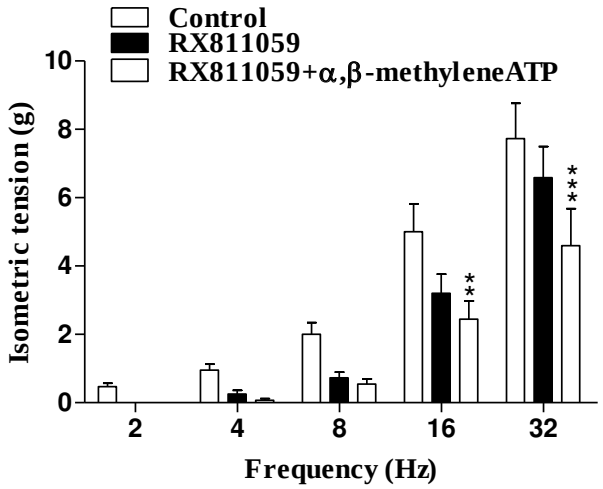



Fig. 4.10 Effects of the sequential addition of RX811059 (1 μM) and α,β-methyleneATP (1 μM) (n=7), on contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30 s, 90 V) in porcine mesenteric first order arteries under raised tone conditions. Each bar represents mean ± standard error. ** $P < 0.01$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

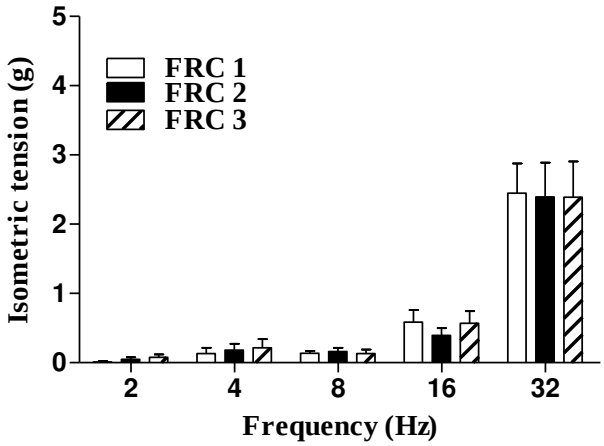



Fig. 4.11 Reproducibility of contractile responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V). Open bars show the first frequency response curve (FRC 1), while the closed bars show the second frequency response curve (FRC 2) and the striped bars show the third frequency response curve (FRC 3) (n=5) in porcine mesenteric third order arteries under basal tone conditions. Each bar represents mean ± standard error.

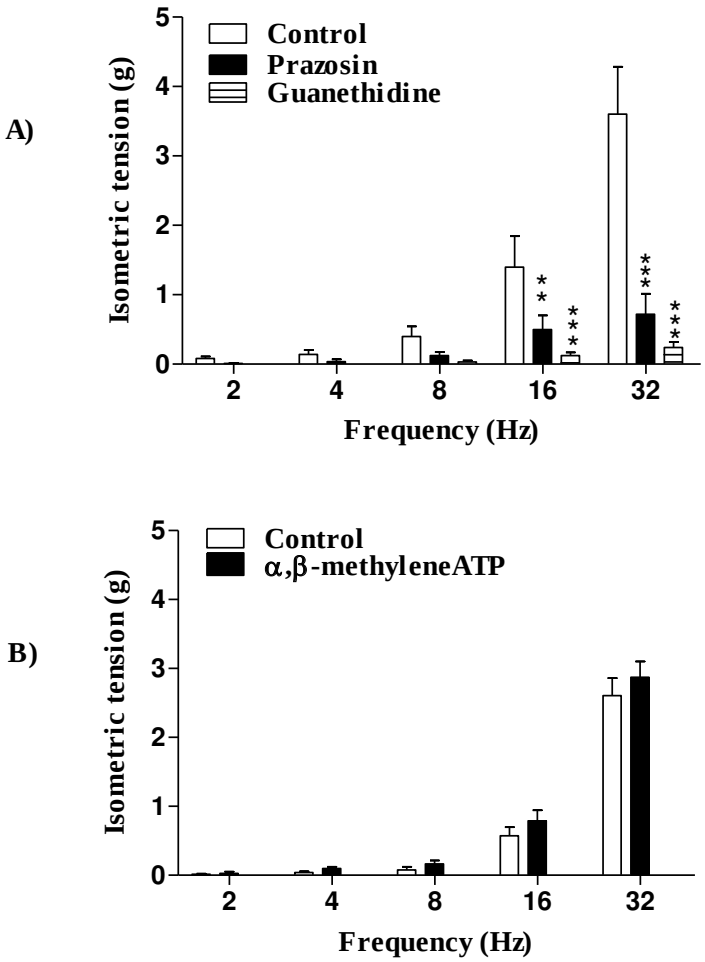



Fig. 4.12 Effects of the sequential addition of A) prazosin (0.1 μM), and guanethidine (1 μM) (n=6), or B) α,β-methyleneATP (1 μM) (n=6), on responses to electrical field stimulation (2-32 Hz, 1 ms, 30 s, 90 V) in porcine mesenteric third order arteries under basal tone conditions. Each bar represents mean ± standard error. ** P < 0.01, *** P < 0.001 vs. control (ANOVA followed by Bonferroni post-hoc test).

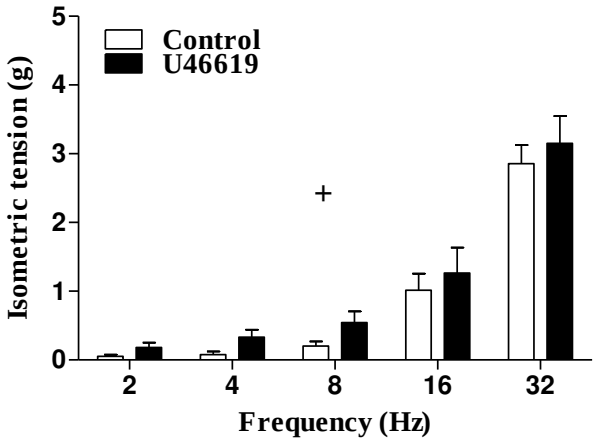



Fig. 4.13 Contractile responses to electrical field stimulation (2-32 Hz, 1 ms, 30s, 90 V) in porcine mesenteric third order arteries under basal tone conditions (control), and in the presence of U46619 (n=5). Each bar represents mean ± standard error. + shows a significant difference between curves ($P < 0.05$, ANOVA followed by Bonferroni post-hoc test).

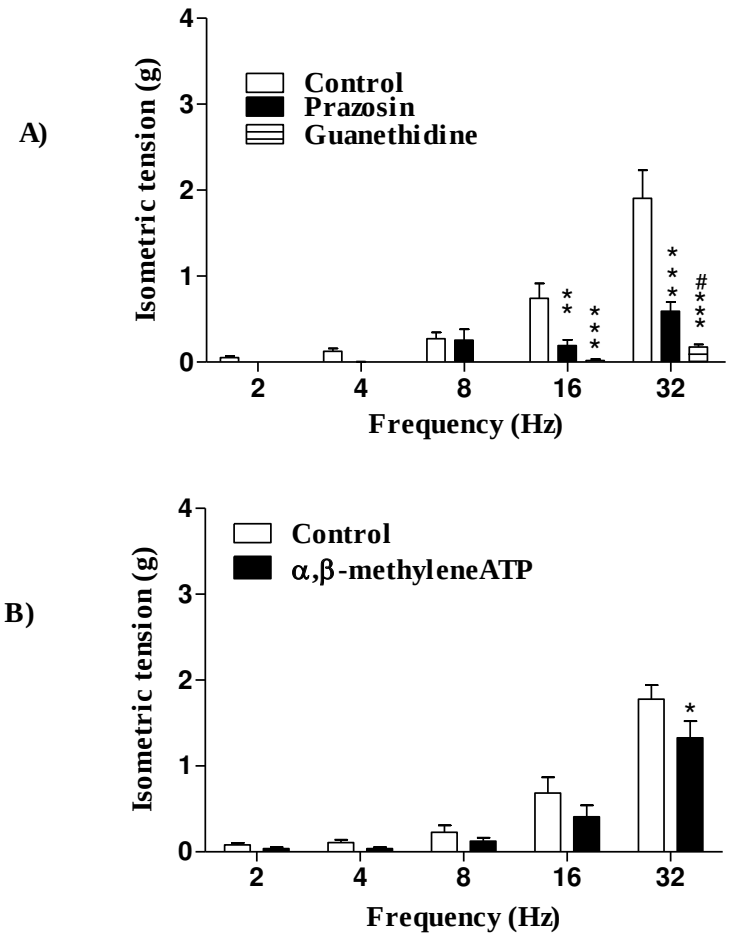



Fig. 4.14 Effects of A) the sequential addition of prazosin (0.1 μM), and guanethidine (1 μM) (n=6), or B) α,β-methyleneATP (1 μM) (n=7), on responses to electrical field stimulation (2-32 Hz, 1ms, 30s, 90 V) in porcine mesenteric third order arteries under raised tone conditions. Each bar represents mean ± standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. # $P < 0.001$ vs. prazosin (ANOVA followed by Bonferroni post-hoc test).

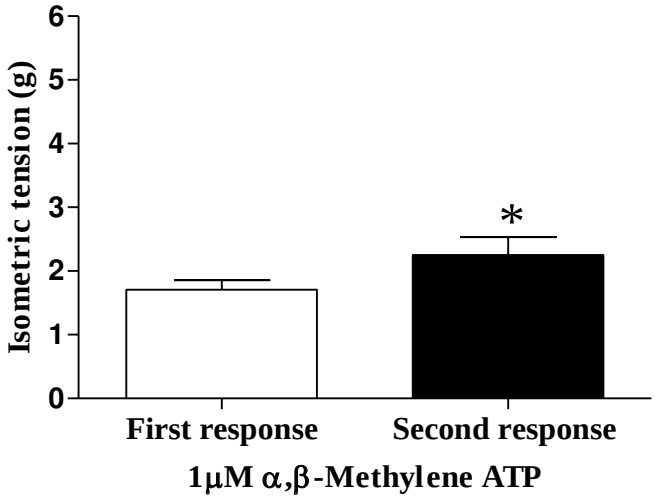
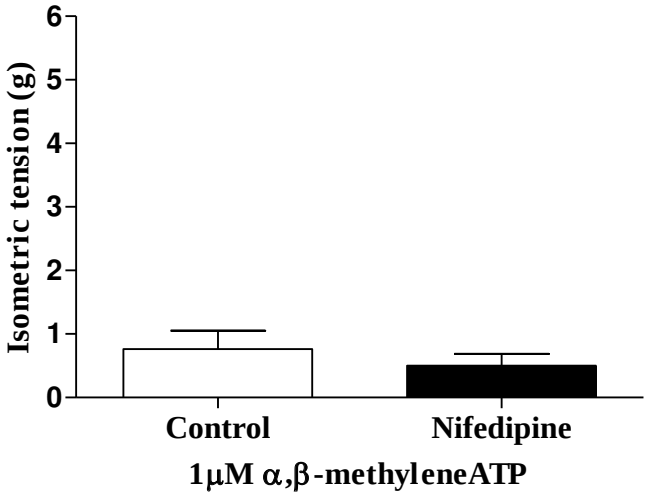



Fig. 4.15 Responses to α,β-methyleneATP (1 μM) (n=8) in porcine mesenteric first order arteries under basal tone conditions. Each bar represents mean ± standard error. * $P < 0.05$ vs. first response (Student's paired t-test).

A)




B)

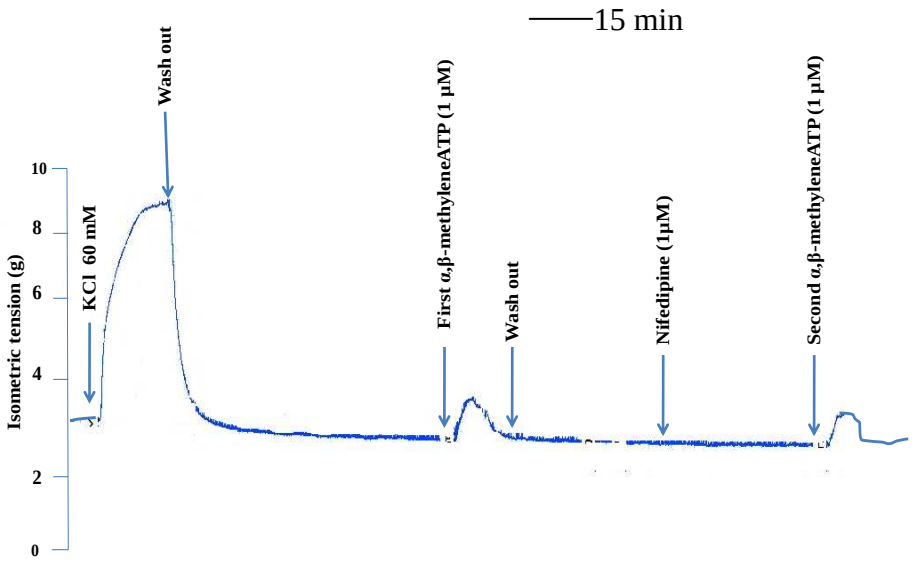



Fig. 4.16 A) Responses to α,β-methyleneATP (1 μM) in the absence of drugs (control), or in the presence of nifedipine (1 μM) (n=8) under basal tone conditions in porcine mesenteric first order arteries. Each bar represents mean ± standard error. B) Representative trace showing responses to α,β-methyleneATP (1 μM) in the absence and in the presence of nifedipine (1 μM) under basal tone conditions in porcine mesenteric first order arteries.

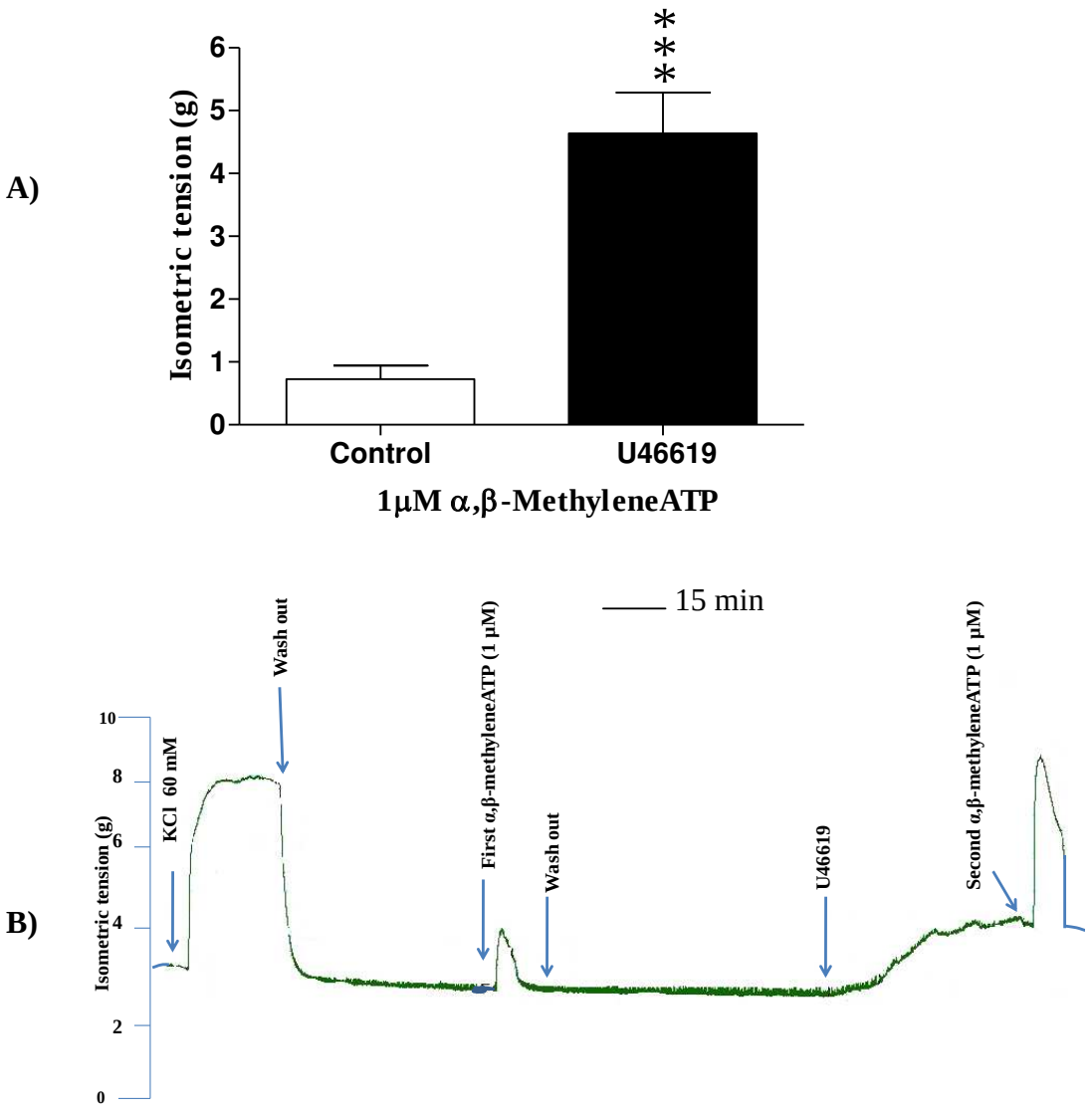



Fig. 4.17 A) Responses to of α,β-methyleneATP (1 μM) under basal tone conditions (control) or in the presence of U46619 (n=8) in porcine mesenteric first order arteries. Each bar represents mean ± standard error. *** P < 0.001 vs. control (Student's paired t-test). B) Representative trace showing the response to α,β-methyleneATP (1 μM) under basal tone conditions and a larger response under conditions of raised tone, in porcine mesenteric first order arteries.

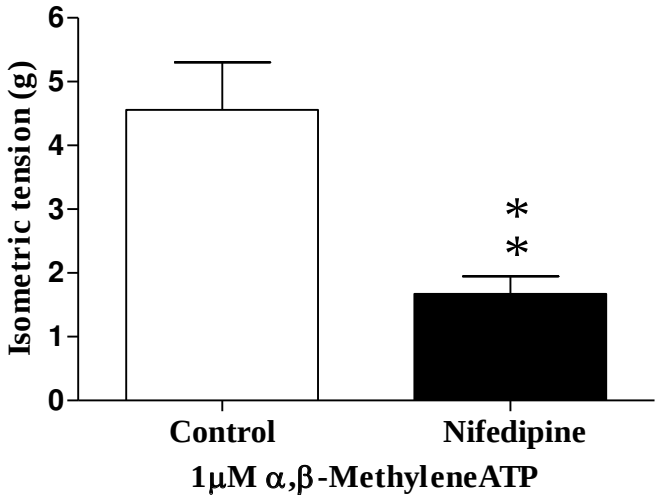



Fig. 4.18 Responses to α,β-methyleneATP (1 μM) under raised tone conditions (control) or in the presence of nifedipine (1 μM) (n=8) in porcine mesenteric first order arteries. Each bar represents mean ± standard error. ** P < 0.01 vs. control (Student's unpaired t-test).

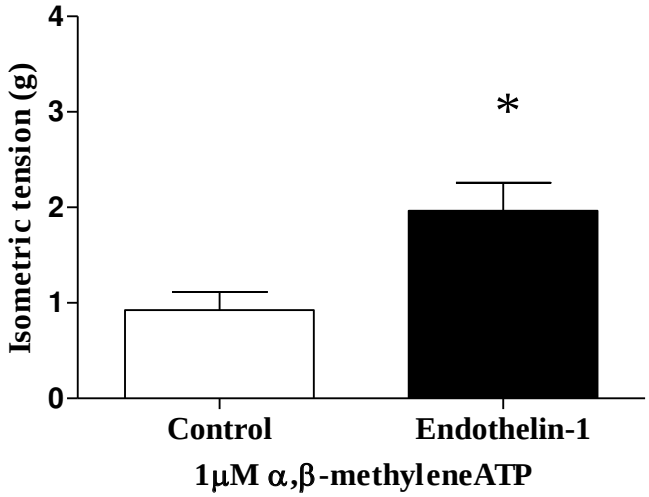
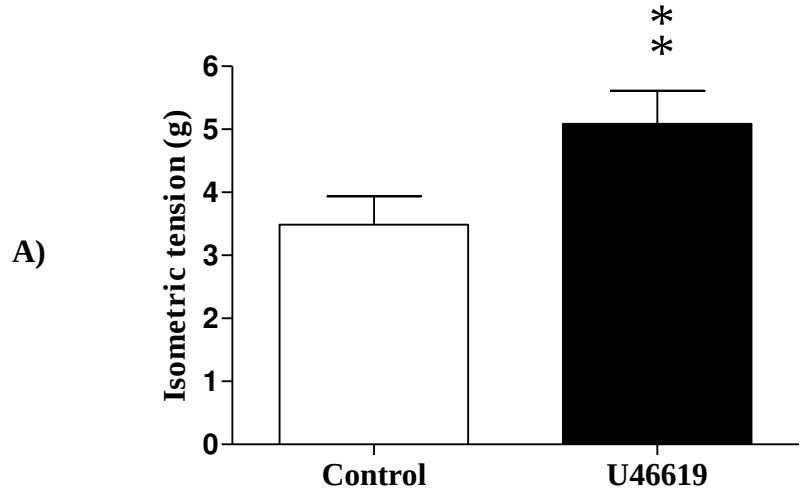



Fig. 4.19 Responses to single application of α,β-methyleneATP (1 μM) under basal tone conditions (control) or in the presence of endothelin-1 (1-2 nM) (n=9) in first order porcine mesenteric arteries. Each bar represents mean ± standard error. * P < 0.5 vs. control (Student's paired t-test).



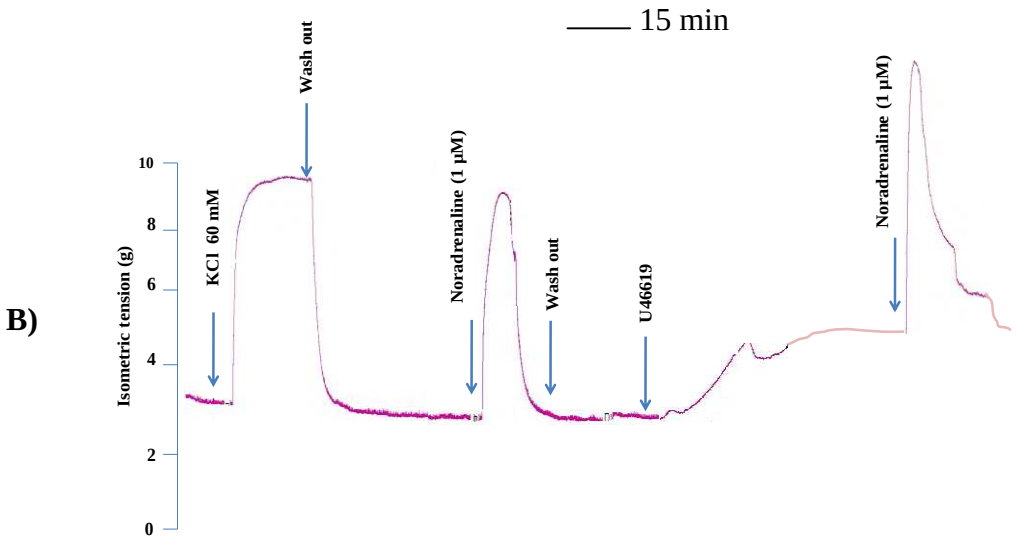
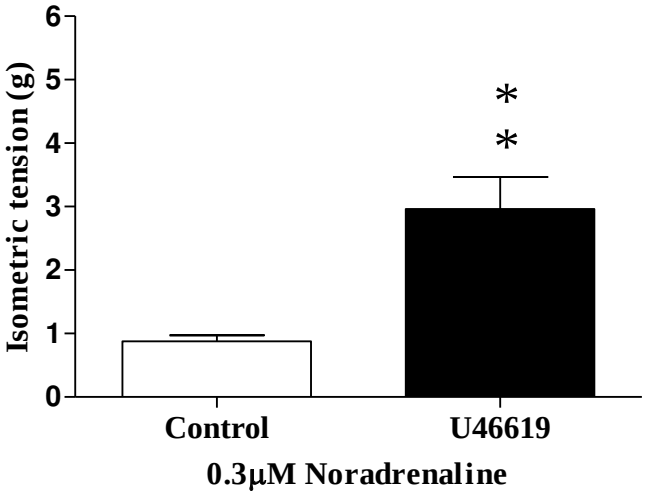




Fig. 4.20 A) Responses to exogenous noradrenaline (1µM) (n=8) under basal tone conditions (control) or in the presence of U46619 (15-20 nM) in porcine mesenteric first order arteries. Each bar represents mean ± standard error. ** $P < 0.01$ vs. control (Student's paired t-test). B) Representative trace showing the effect of single concentration application of NA (1 µM) under basal tone conditions and a larger response under conditions of raised tone, in porcine mesenteric first order arteries.

A)




B)

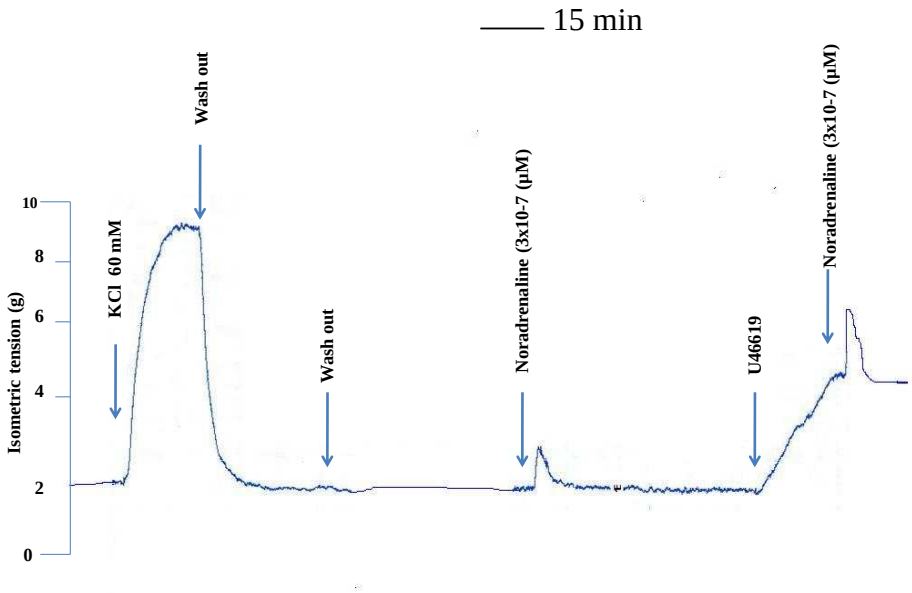



Fig. 4.21 A) Responses to exogenous noradrenaline (0.3 µM) (n=8) under basal tone conditions (control) or in the presence of U46619 (15-25 nM) in porcine mesenteric first order arteries. Each bar represents mean ± standard error. ** P < 0.01 vs. control (Student's paired t-test). B). Representative trace showing the response to noradrenaline (0.3 µM) under basal tone conditions and a larger response under conditions of raised tone, in porcine first order mesenteric arteries.

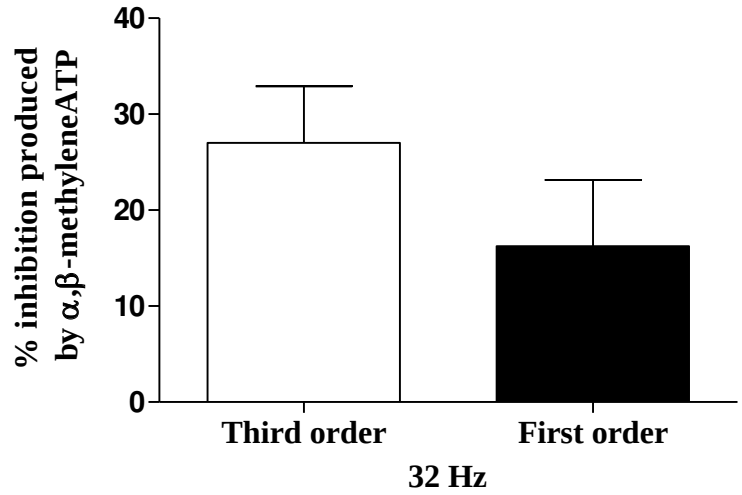



Fig. 4.22 Effects of α,β-methyleneATP (1 μM) on the responses to electrical field stimulation (32 Hz, 1ms, 30s, 90 V) in porcine mesenteric third order and first order arteries under raised tone conditions (n=9). Each bar represents mean ± standard error. P > 0.05 (Mann-Whitney test).

## 4.5 DISCUSSION

The main finding of the present chapter is, that under conditions in which tone was raised with U46619, ATP has contributed to the mediation of the electrically-evoked contractile response in porcine first and third order mesenteric arteries. In contrast ATP had no involvement under basal tone conditions. Responses to α,β-methyleneATP and exogenous NA were enhanced under raised tone conditions indicating the involvement of a postjunctional mechanism in the larger responses seen under raised tone conditions.

First order arteries

In porcine first order mesenteric arteries, under basal tone conditions electrically-evoked contractions were reproducible. TTX and guanethidine virtually abolished the electrically-evoked contractile responses, indicating that the responses were neurogenic and sympathetic in origin. Prazosin inhibited the electrically-evoked contractile responses to a similar extent to TTX or guanethidine, while α,β-methyleneATP did not alter the responses. These observations show that NA is the main sympathetic neurotransmitter acting via $\alpha_1$-adrenoceptors in porcine mesenteric first order arteries under basal tone conditions.

Under raised tone conditions, the nerve-mediated responses were slightly enhanced. Guanethidine virtually abolished the electrically-evoked contractile responses in porcine mesenteric first order arteries, under these conditions. In contrast, there was a significant residual component remaining in the presence

of prazosin. This suggests that another transmitter or receptor mechanism is functional under raised tone conditions. One possibility is $\alpha_2$-adrenoceptors. These receptors have been shown to be involved in mediating postjunctional effects of neuronally-derived NA, but only in the presence of an unrelated agonist such as angiotensin II, as shown for example in the rabbit distal saphenous artery (Dunn et al., 1991b). The data obtained in the present study indicate some involvement of $\alpha_2$-adrenoceptors in mediate the neurogenic contractile responses in porcine mesenteric arteries under both raised and basal tone conditions, since RX811059, an $\alpha_2$-adrenoceptor antagonist, inhibited the electrically-evoked contractile responses. However, the addition of RX811059 after prazosin under raised tone conditions did not affect the prazosin-resistant component of the response. It should be noted that the interpretation of RX811059 effects might be complicated by its possible prejunctional actions (disinhibition of neurotransmitter release) which might lead to an underestimation of the role of postjunctional $\alpha_2$-adrenoceptors in causing contraction.

In chapter 3 I demonstrated that ATP was involved in mediation of the electrically-evoked vasoconstrictor responses in the porcine perfused bed under raised tone conditions. Thus, to investigate the involvement of ATP, α,β-methyleneATP, a $P2X_1$ receptor desensitising agent, was used. α,β-methyleneATP had a significant inhibitory effect on responses to EFS only in the presence of U46619, while under basal tone conditions it had no significant effect. This indicates that while NA is the main neurotransmitter acting mostly through $\alpha_1$-adrenoceptors, ATP contributes to part of the response through

activation of $P2X_1$ receptors, under raised tone conditions only. This is essentially the same observation as made in the perfused mesenteric bed (see chapter 3).

The other possibility is an altered function of NPY as a sympathetic co-transmitter under raised tone conditions (Lundberg et al., 1983). Raising the tone can enhance NPY responses, for example, small responses to exogenous NPY were obtained under basal tone conditions which were enhanced in the presence of U46619 in porcine isolated ear artery (Roberts et al., 1999). However, the NPY $Y_1$ receptor antagonist BIBP3226 had no significant effect on electrically-evoked contractile responses under conditions of raised tone indicating that $Y_1$ receptors were not involved in the enhanced nerve response in porcine mesenteric arteries. This perhaps is not surprising since, for the most part, NPY acts as a neuromodulator with little or no postjunctional actions, although in some vessels, especially in cerebral arteries, it can produce vasoconstrictor responses (Fredholm et al., 1985, Edvinsson et al., 1994). Thus, it seems that porcine mesenteric arteries differ from cerebral arteries in terms of the involvement of NPY as a sympathetic neurotransmitter. These data demonstrate that under raised tone conditions, NA mediates contractions by activating postjunctional $\alpha_1$-adrenoceptors and $\alpha_2$-adrenoceptors and that ATP via P2X receptors is also involved in mediating sympathetic-nerve mediated responses in these vessels.

EFS can stimulate not only sympathetic nerves but also sensory nerves. Activation of sensory nerves can cause vasodilatation in some vascular beds by releasing different neurotransmitters principally calcitonin gene-related peptide

(CGRP) (Kawasaki et al., 1988). Capsaicin acts by stimulating transient receptor potential vanilloid receptor type 1 (TRPV1) receptors located on capsaicin-sensitive sensory nerves (Gupta et al., 2007). The exposure of sensory nerves to capsaicin desensitizes and depletes them of their neurotransmitters (Maggi and Meli, 1988, Caterina and Julius, 2001). In the present study pre-treatment with capsaicin had no significant effect on the size of the electrically-evoked contractile responses in porcine mesenteric first order arteries. These results are similar to those obtained in the porcine perfused bed (see chapter 3) but different from those obtained in the rat perfused mesenteric bed. In the rat perfused mesenteric bed EFS produced a two-phase response vasoconstrictor and vasodepressor responses (Pakdeechote et al., 2007). Pre-treatment with capsaicin abolished the vasodepressor response and enhanced the electrically-evoked vasoconstrictor responses indicating an involvement of sensory nerves (Pakdeechote et al., 2007). The simple explanation could be a difference between the species.

Third order arteries

A very similar pattern to that seen with first order vessels was seen in third order arteries. Under basal tone conditions, electrically-evoked contractions were reproducible and sympathetic in origin since guanethidine almost abolished them. Prazosin had a similar effect to that of guanethidine, indicating that electrically-evoked contractions were mediated by NA acting through postjunctional $\alpha_1$-adrenoceptors. Furthermore α,β-methyleneATP had no effect on electrically-evoked contractile responses indicating that P2X receptors were not involved in the mediation of these responses.

U46619 enhanced the nerve-mediated responses. In the presence of U46619, guanethidine was more effective at blocking responses than prazosin especially at higher frequencies, showing that while NA was still the major neurotransmitter, acting via $\alpha_1$-adrenoceptors, some other mechanism was involved. This was most likely ATP, since α,β-methyleneATP significantly inhibited the electrically-evoked contractile responses in third order arteries in the presence of U46619.

Data obtained in the present study showed that responses to exogenous NA or, the analogue of ATP, α,β-methyleneATP, were enhanced under raised tone conditions. Furthermore, our results showed that responses to α,β-methyleneATP were enhanced not only in the presence of U46619 but also in the presence of endothelin-1. These results are consistent with the results obtained in the rat perfused mesenteric bed where responses to α,β-methyleneATP and EFS were enhanced in the presence of either U46619 or endothelin-1 (Pakdeechote et al., 2007). Thus, enhancement not specific to U46619 but a consequence of the increase in tone.

The fact that the overall response to EFS gets bigger is due, at least in part, to the uncovering of a purinergic response as evidenced by the inhibitory effect of α,β-methylene at raised but not at basal tone conditions and by the enhanced contractions to α,β-methylene. P2X receptor activation leads to a direct influx of cations into the cell and depolarization of the smooth muscle leading to the opening of voltage-sensitive calcium channels (Lagaud et al., 1996). Voltage-sensitive calcium channels have been shown to be involved in mediating purinergic vasoconstriction in blood vessels including canine mesenteric

arteries and rabbit ileocolic arteries (Omote et al., 1989, Bulloch et al., 1991). To investigate the role of voltage-sensitive calcium channels in the mediation of the responses to α,β-methyleneATP, the L-type calcium channel blocker nifedipine was used. Nifedipine had no significant effect on responses to α,β-methyleneATP under conditions of basal tone but reduced responses after raising tone with U46619. This may indicate that voltage-sensitive calcium channels were involved in P2X receptors-mediated contractions only under conditions of raised tone. It has been demonstrated that U46619 depolarizes vascular smooth muscle (Crane and Garland, 2004, Shaw et al., 2004) and this may provide conditions where further depolarization caused by ATP acting on P2X receptors results in further opening of L-type $Ca^{2+}$ channels and thus contraction. The change in membrane potential may explain the enhanced response and the uncovering of a purinergic response under raised tone conditions as described in pressurised arteries (Rummery et al., 2007).

In the current study there was an enhancement of responses to exogenous NA in pre-constricted arteries. It is possible that this enhancement may be due to an the additional involvement of postjunctional $\alpha_2$-adrenoceptors under raised tone conditions, since $\alpha_2$-adrenoceptors have been shown to be involved in mediating postjunctional effects of neuronally-derived NA, especially in the presence of an agonist such as angiotensin II, in the rabbit distal saphenous artery (Dunn et al., 1989, Dunn et al., 1991b) and parallels results obtained in this present study where there was some evidence for an involvement of $\alpha_2$-adrenoceptors in neurogenic responses at raised tone. Another possibility is the involvement of voltage-sensitive calcium channels in the mediation of the

enhanced noradrenergic response since there is evidence supporting the dependence of $\alpha_2$-adrenoceptors upon an influx of extracellular calcium through voltage-sensitive calcium channels (McGrath et al., 1989, Dunn et al., 1991a).

One of the aims in studying first and third order arteries was to determine if the role of ATP as a sympathetic neurotransmitter increased as arteries got smaller (Gitterman and Evans, 2001). However, a comparison between the effect of α,β-methyleneATP on the electrically-evoked contractile responses in first and third order arteries showed no difference. This may indicate that although first and third order arteries have a purinergic component, other parts of the vascular tree (smaller arteries) may be involved in the uncovering of the purinergic response observed at raised tone in the whole bed preparation, since the purinergic component shown in first and third arteries under raised tone conditions is modest compared to that obtained in the porcine perfused bed at raised tone. Thus, chapter 5 studied smaller arteries using isometric myograph techniques.

In conclusion the present study has shown that, in porcine first and third order mesenteric arteries, pre-constriction with U46619 uncovered the involvement of ATP as a neurotransmitter acting through P2X receptors, which was not evident under basal tone conditions, while NA acts as the main neurotransmitter acting principally via postjunctional $\alpha_1$-adrenoceptors with a smaller contribution of $\alpha_2$-adrenoceptors both under basal and raised tone conditions.

# CHAPTER 5

# CHARACTERIZATION OF SYMPATHETIC NEUROTRANSMISSION IN PORCINE SMALL MESENTERIC ARTERIES

## 5.1 INTRODUCTION

In chapter 4 I demonstrated a small purinergic component in the response to EFS in porcine first and third order mesenteric arteries under raised tone conditions. However, the size of the purinergic component uncovered by raising tone in the porcine perfused bed described in chapter 3, was more substantial indicating that some other part of the vascular tree comprising the mesenteric arterial bed was more probably contributing. Furthermore, it has been demonstrated that the purinergic component of responses to nerve stimulation becomes more important in smaller arteries (Gitterman and Evans, 2001).

The aim of the present chapter was to characterize sympathetic neurotransmission in the porcine mesenteric small resistance arteries using wire myography.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Tissue preparation for immunohistochemical staining

Porcine mesenteries were obtained as described in section 2.1. Fine dissection was carried out to identify small terminal branch arteries using a dissecting

microscope. Porcine mesenteric small arteries were then used for either immunohistochemical staining as described in section 2.3 or wire myography as described in section 2.4.

### 5.2.2 Response to EFS in porcine mesenteric small arteries under basal tone conditions

After an equilibration period of 60 min responses to EFS (2-16 Hz, 1 ms, 10 V, 30 s) were obtained. The interval between each frequency was variable (2-4 min), and was determined by the return to baseline after each stimulation. Preparations were exposed to prazosin (0.1 μM), after the first FRC, while the combination of prazosin (0.1 μM) plus α,β-methyleneATP (1 μM) was added after the second FRC and a third FRC was constructed. All antagonist drugs were left for 30 min before testing their effect on the electrically-evoked vasoconstrictor responses.

### 5.2.3 Response to EFS in porcine mesenteric small arteries under raised tone conditions

After an equilibration period of 60 min, prior to exposing the tissue to EFS, U46619 (0.5 - 2 nM), a thromboxane $A_2$ agonist, was used to raise the tone to about 25% of the second KCl response. Responses to EFS (2-16 Hz, 1 ms, 10 V, 30 s) were then determined. In some preparations a FRC was obtained under basal tone conditions after which U46619 was added to raise the tone and a second FRC was conducted. Other preparations were exposed to prazosin (0.1 μM) after the first FRC, while a combination of prazosin (0.1 μM) plus α,β-methyleneATP (1 μM) was added after the second FRC and a

third FRC was constructed. The order of drug addition was changed in other preparations.

In some experiments the L-type calcium channel blocker nifedipine (1 µM) (Blakeley et al., 1981, Rummery et al., 2007) was added after the second FRC before a third FRC was constructed. Antagonist drugs were left for 30 min before testing their effect on the electrically-evoked contractile responses. In experiments where nifedipine was used the vessels were pre-constricted with U46619 to about 35-45% of the second KCl response. Thus, when nifedipine was added, the tone of pre-constricted vessels decreased back to 25% of the second KCl response. Only after reaching a stable level of pre-constriction, was a further FRC constructed.

### 5.2.4 Responses to exogenous noradrenaline and α,β-methyleneATP in porcine mesenteric small arteries under basal and raised tone conditions

To assess vascular responsiveness a single concentration of NA (1µM) or α,β-methyleneATP (1 µM) was obtained under basal tone conditions. The tissue was washed twice with Krebs-Henseleit buffer for 30 min and U46619 (0.5-2 nM) was then added to pre-constrict the vessel to about 25% of the second KCl response. When a stable contraction to U46619 was achieved, a second application of NA (1µM) or α,β-methyleneATP (1 µM) was applied. Similar experiments were conducted in large arteries.

## 5.3 STATISTICAL ANALYSIS

Results are expressed as the mean ± S.E.M. Statistical comparisons were made by two way analysis of variance (ANOVA) with Bonferroni post-hoc test or Student's paired t-test if the data were normally distributed (checked by Shapiro-Wilk normality test) while Mann-Whitney test was used if the data were not normally distributed. A value of $P < 0.05$ was taken to indicate statistical significance.

## 5.4 RESULTS

### 5.4.1 Immunohistochemical characterization of perivascular nerves in porcine mesenteric small arteries

The general neuronal marker protein gene product 9.5 (PGP9.5), a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate ubiquitin monomer, which is highly expressed in neurons, was used to visualize nerves within porcine mesenteric small arteries. Nerves immunoreactive to PGP9.5 were identified in porcine mesenteric small arteries (Fig. 5.1A), while the controls, where primary antibody was not applied, showed no binding of the secondary antibody (Fig. 5.1B).

Tyrosine hydroxylase (TH) is an enzyme involved in the conversion of tyrosine to dopamine in the synthesis of catecholamines in sympathetic neurons. TH marker was used to visualize noradrenergic nerves within small porcine mesenteric arteries. Nerves immunoreactive to TH were identified in small porcine mesenteric arteries (Fig. 5.2A), while the controls, where

primary antibody was not applied, showed no immunoreactivity to TH (Fig. 5.2B).

### 5.4.2 Effects of prazosin and α,β-methyleneATP in porcine mesenteric small arteries under basal tone conditions

Under basal tone conditions EFS produced contractile responses which were frequency-dependent. α,β-methyleneATP (1 μM) caused a transient contraction (0.80 ± 0.07 g, n=7) which returned to the baseline before the construction of the next FRC, but had no significant effect on the contractile responses to EFS in porcine mesenteric small arteries under basal tone conditions (n=7) (Fig. 5.3). Subsequent exposure to prazosin (0.1 μM) significantly inhibited the contractile responses to EFS ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 5.3).

### 5.4.3 Effects of prazosin and α,β-methyleneATP in porcine mesenteric small arteries under raised tone conditions

U46619 (0.5-2 nM) contracted porcine mesenteric small arteries by 20 ± 3% of KCl response (n=14). Under raised tone conditions, EFS produced contractile responses which were frequency-dependent and larger than under basal tone conditions at all frequencies reaching statistical significance at 16 Hz ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=12) (Fig. 5.4).

α,β-methyleneATP (1 μM), a P2X receptor desensitizing agent, caused a transient contraction (0.95 ± 0.5 g, n=7) which returned to the baseline before the construction of the next FRC. α,β-methyleneATP inhibited the responses to

nerve stimulation (e.g. at 8 Hz by 41 ± 11% and at 16 Hz by 36 ± 8%) (Fig. 5.5A) in porcine mesenteric small arteries under raised tone conditions, in contrast to its lack of effect under basal tone conditions (see Fig. 5.3). Further addition of prazosin (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, abolished the response at 2, 4 and 8 Hz leaving a small residual response at 16 Hz ($P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 5.5A) presumably due to direct smooth muscle activation.

When prazosin was added first it almost abolished the response at lower frequencies leaving a residual response at higher frequencies which was reduced by further addition of α,β-methyleneATP (n=5) (Fig. 5.5B).

### 5.4.4 Effects of nifedipine in porcine mesenteric small arteries under raised tone conditions

The L-type calcium channel blocker nifedipine (1 μM), decreased the raised tone that was produced by U46619 by 15-20% and prevented the enhanced responses produced in the presence of U46619 at all frequencies ($P < 0.01$, ANOVA followed by Bonferroni post-hoc test, n=4) (Fig. 5.6), such that in the presence of nifedipine responses to EFS were similar to contractile responses under basal tone conditions.

### 5.4.5 Effects of exogenous noradrenaline in porcine mesenteric small arteries under basal and raised tone conditions

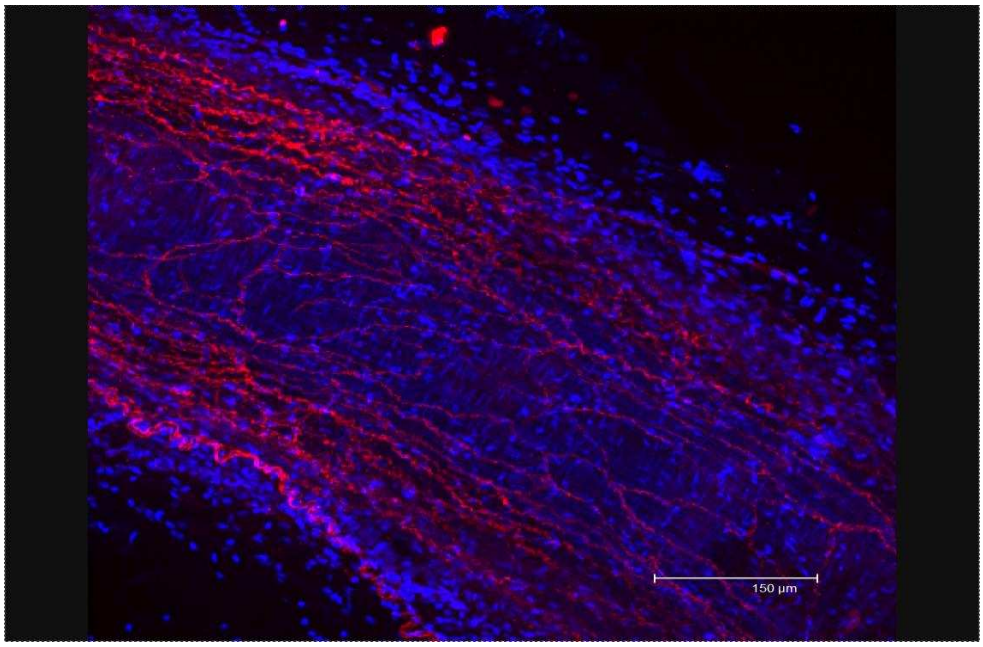
Responses to exogenous NA (1 μM) were enhanced under raised tone conditions (n=4) Mann-Whitney test (Fig. 5.7).

### 5.4.6 Comparison of response to α,β-methyleneATP (1 µM) in porcine first order and small mesenteric arteries under basal and raised tone conditions

KCl was used to check the viability and the maximum contractility of vascular tissue. KCl (60 mM) contracted both porcine mesenteric small arteries (n=22) (Fig. 5.8) and porcine mesenteric first order arteries (n=16) (Fig. 5.9).

Responses to α,β-methyleneATP (1 µM) were significantly enhanced in the presence of U46619 in porcine mesenteric small arteries ($P < 0.01$, one way ANOVA) (n=11) (Fig. 5.8). Similarly, responses to α,β-methyleneATP (1 µM) were enhanced in the presence of U46619 in porcine mesenteric first order arteries (*** $P < 0.001$, one way ANOVA) (n=8) (Fig. 5.9).

**A)**




**B)**

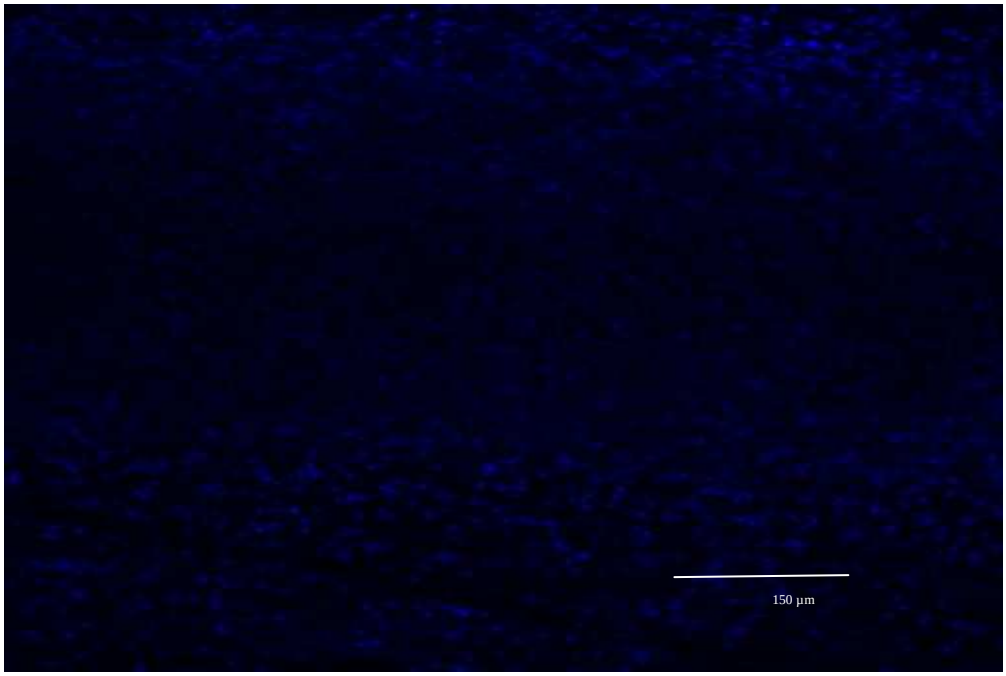
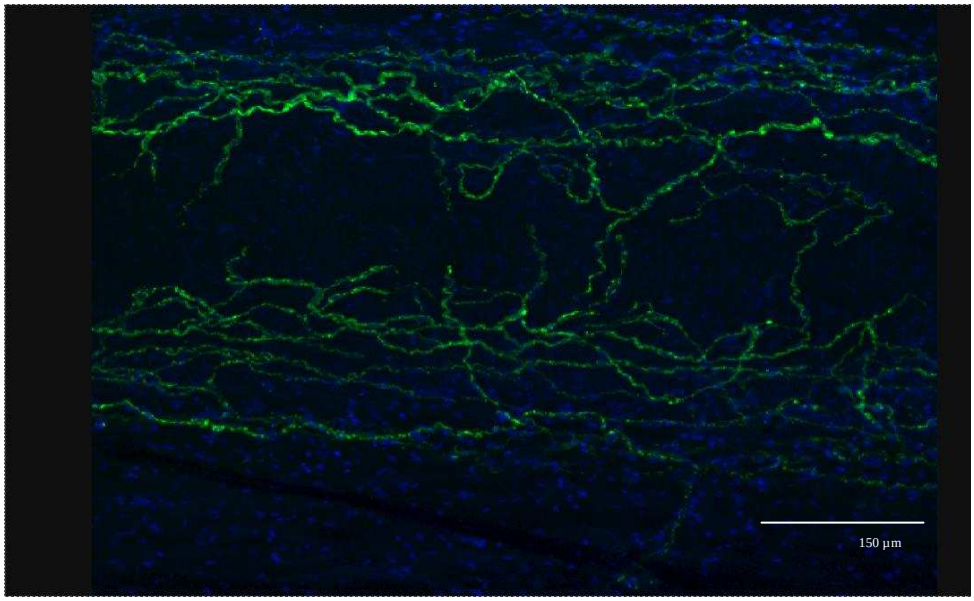



Fig. 5.1 A representative of maximum projection confocal images of whole-mount preparations of porcine mesenteric small arteries. A) Immunoreactive perivascular nerves stained for PGP9.5 (red) can be seen. B) When PGP9.5 (primary antibody) was not applied (control) perivascular nerves cannot be identified. Scale bar = 150 µm.

**A)**

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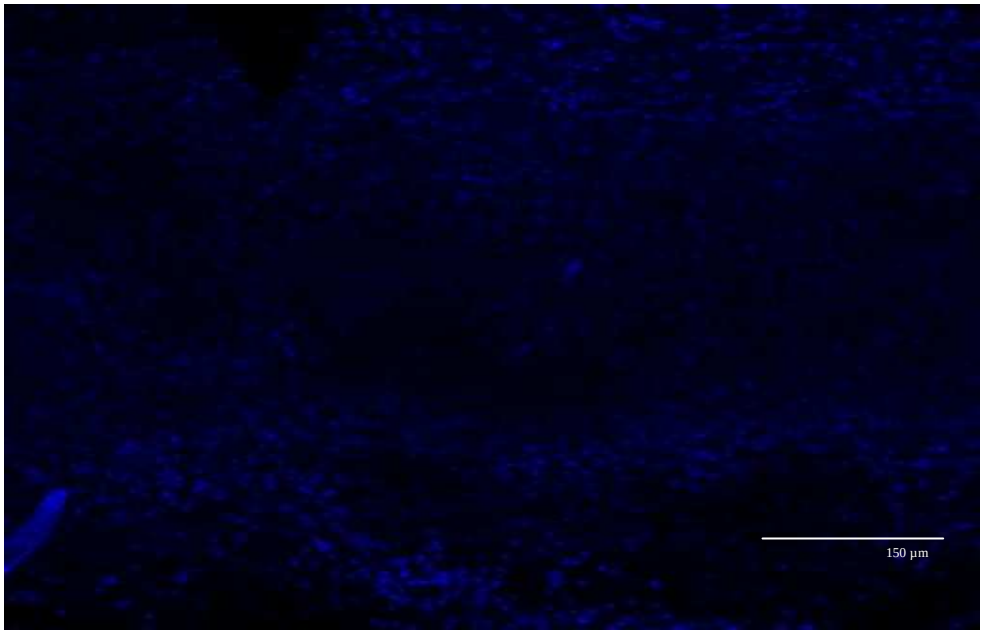


Fig. 5.2 A representative of maximum projection confocal images of whole-mount preparations of porcine mesenteric small arteries. A) Immunoreactive perivascular nerves stained for TH (green) can be seen. B) When TH (primary antibody) was not applied (control) perivascular nerves cannot be identified. Scale bar = 150 µm.

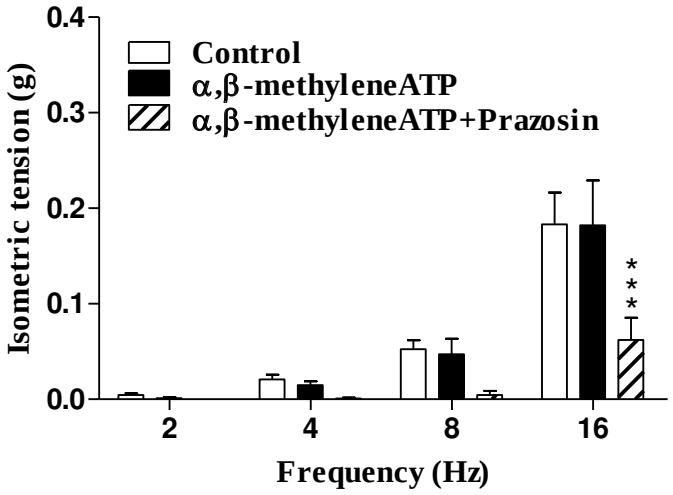


Fig. 5.3 Effects of the sequential addition of α,β-methyleneATP (1 μM) followed by prazosin (0.1 μM) (n=7) under basal tone conditions, on responses to electrical field stimulation (2-16 Hz, 1 ms, 30 s, 10V) in porcine mesenteric small arteries. Each bar represents mean ± standard error. *** $P < 0.001$ vs. α,β-methyleneATP (ANOVA followed by Bonferroni post-hoc test).

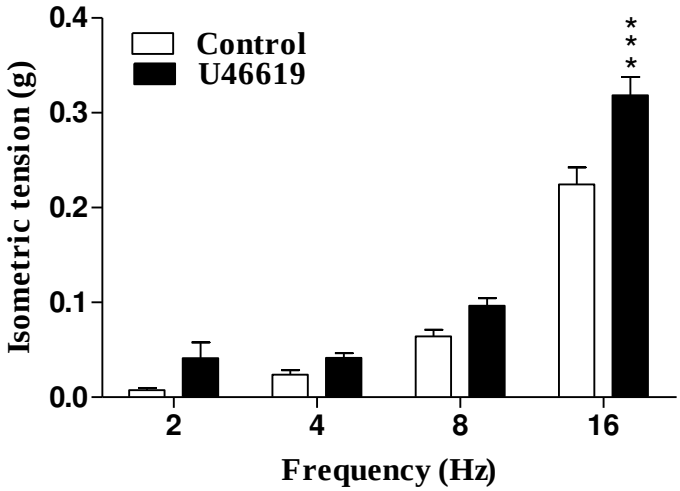


Fig. 5.4 Contractile responses to electrical field stimulation (2-16 Hz, 1 ms, 30 s, 10V) in porcine mesenteric small arteries under basal tone conditions (control), and in the presence of U46619 (n=12). Each bar represents mean ± standard error. *** P < 0.001 vs. control (ANOVA followed by Bonferroni post-hoc test).

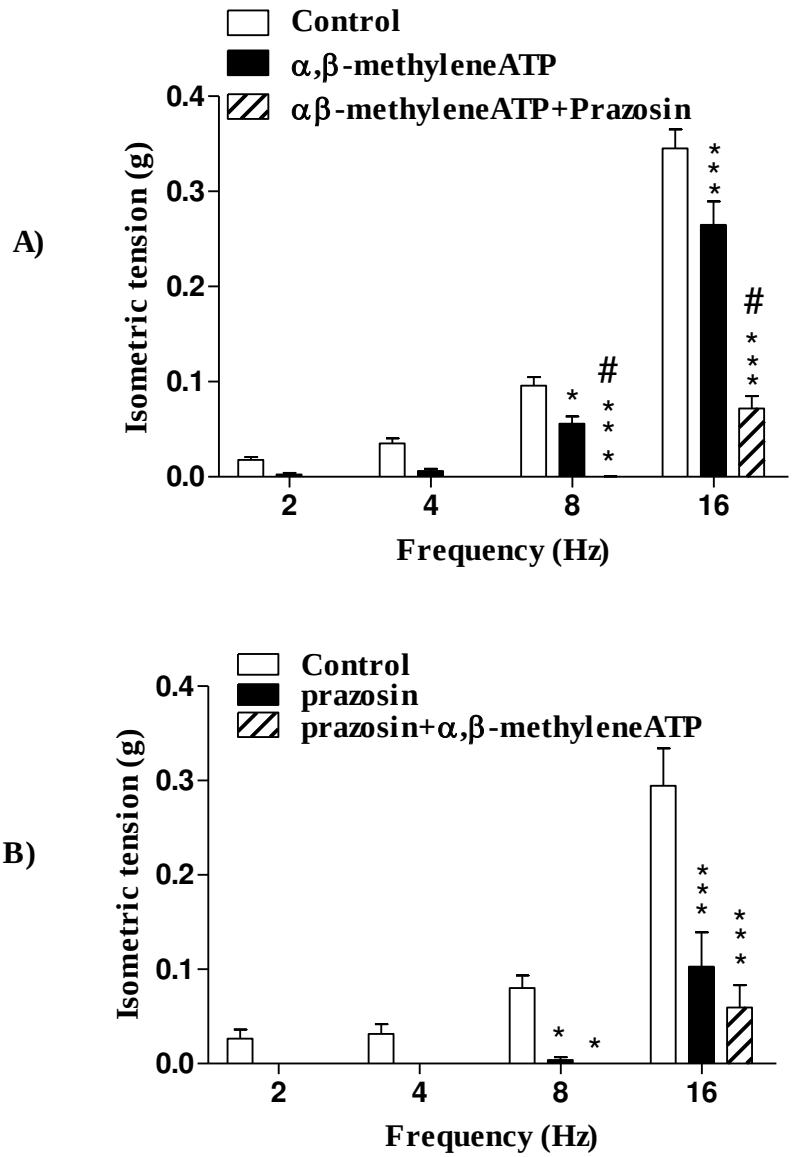


Fig. 5.5 Effects of the sequential addition of A) α,β-methyleneATP (1 μM) followed by prazosin (0.1 μM) (n=7), or B) prazosin (0.1 μM) followed by α,β-methyleneATP (1 μM) (n=5) under raised tone conditions, on responses to electrical field stimulation (2-16 Hz, 1 ms, 30 s, 10V) in porcine mesenteric small arteries. Each bar represents mean ± standard error. * $P < 0.05$, *** $P < 0.001$ vs. control. # $P < 0.001$ vs. α,β-methyleneATP (ANOVA followed by Bonferroni post-hoc test).

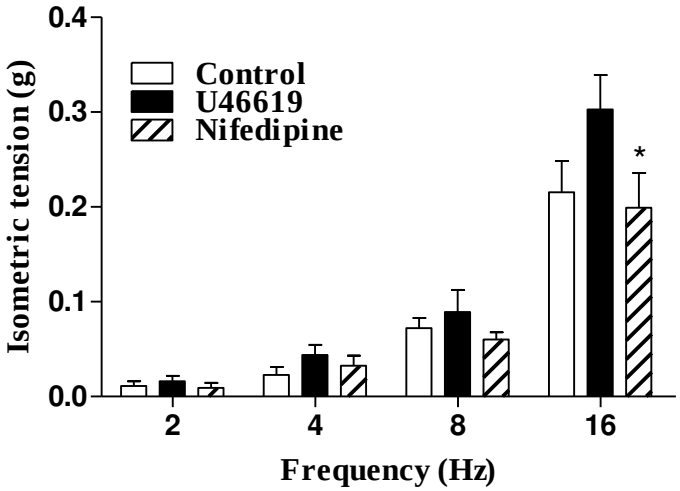


Fig. 5.6 Contractile responses to electrical field stimulation (2-16 Hz, 1 ms, 30 s, 10V) in porcine mesenteric small arteries under basal tone conditions (control), in the presence of U46619 or in the presence of nifedipine (1 μM) (n=4). Each bar represents mean ± standard error. * $P < 0.01$ vs. U46619 (ANOVA followed by Bonferroni post-hoc test).

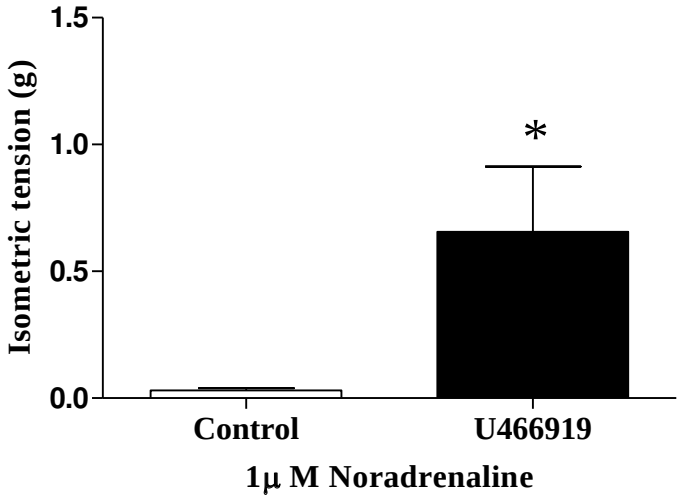


Fig. 5.7 Responses to exogenous noradrenaline (1 μM) (n=4), under basal tone conditions (control) or in the presence of U46619-induced tone in porcine mesenteric small arteries. Each bar represents mean ± standard error. * $P < 0.05$ vs. control (Mann Whitney test).

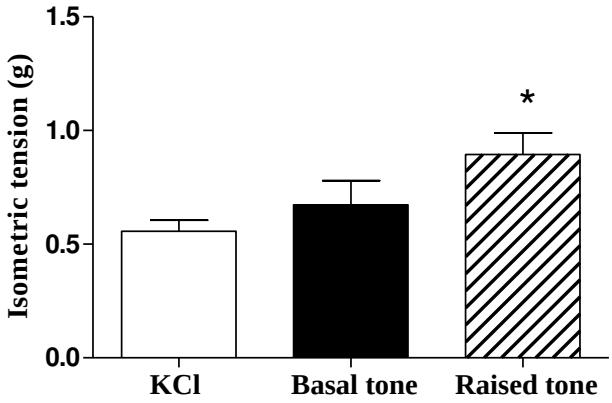


Fig. 5.8 Responses to KCl (60 mM) (n=22) (basal tone) and α,β-methyleneATP (1 μM) (n=11) under basal or raised tone conditions in porcine mesenteric small arteries. Each bar represents mean ± standard error. * $P < 0.05$ vs. basal tone conditions (one way ANOVA).

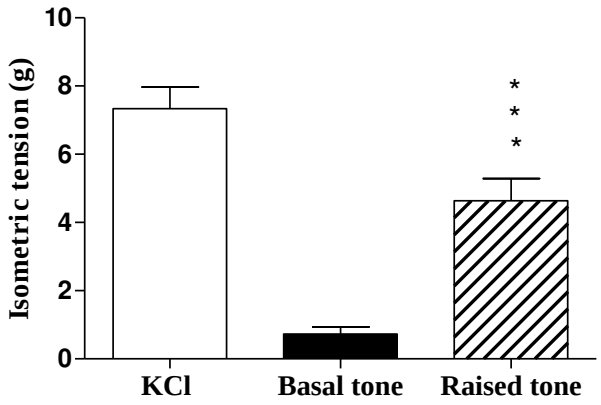


Fig. 5.9 Responses to KCl (60 mM) (basal tone) (n=16) and α,β-methyleneATP (1 μM) (n=8) under basal or raised tone conditions in porcine mesenteric first order arteries. Each bar represents mean ± standard error. *** $P < 0.001$ vs. basal tone conditions (one way ANOVA).

## 5.5 DISCUSSION

Immunohistochemical staining of porcine mesenteric small arteries showed the presence of substantial innervation with perivascular nerves where fibres immunoreactive to PGP 9.5 were identified. Staining with TH revealed an abundance of sympathetic nerves among these perivascular nerves. Similar observations have been reported in human mesenteric arteries where immunoreactive fibres at the adventitial-medial border were identified (Birch et al., 2008).

Functional studies of the porcine mesenteric small arteries obtained in the present study have demonstrated that ATP is an important sympathetic neurotransmitter in porcine mesenteric small arteries. This was apparent since α,β-methyleneATP inhibited the nerve-mediated response under conditions of raised tone only, in contrast to the lack of effect of α,β-methyleneATP under basal tone conditions, where nerve-mediated responses were resistant to α,β-methyleneATP but almost completely abolished by prazosin. These results indicate that ATP is not involved as a sympathetic cotransmitter under basal tone conditions and most of the response was mediated by NA acting via postjunctional $\alpha_1$-adrenoceptors.

The resistant to α,β-methyleneATP under basal tone conditions in the current study has also been observed in the porcine perfused arterial bed, and first and third order arteries, but contradict another observation in rat small mesenteric arteries under basal tone conditions, where ATP has been shown to be the dominant sympathetic neurotransmitter (Gitterman and Evans, 2001). While

this may be explained as a difference between species a more likely explanation is that different experimental conditions employed may affect the purinergic component of the sympathetic response, since in the rat perfused mesenteric arterial bed there was no role for ATP as a sympathetic cotransmitter under basal tone conditions tone (Pakdeechote et al., 2007).

Under conditions of raised tone using U46619, the nerve-mediated responses became larger. Furthermore responses to exogenously applied NA and α,β-methyleneATP were enhanced. This suggests a postjunctional mechanism of enhancement to both components of sympathetic neurotransmission; noradrenergic and purinergic. These observations have also been shown in studies where U46619 enhanced the responses to nerve-mediated and exogenously applied NA in human saphenous vein (Vila et al., 2001). Responses to α,β-methyleneATP have also been enhanced in the presence of U46619 in the rat perfused mesentery (Pakdeechote et al., 2007). Moreover, these results parallel my observations in chapter 4 in isolated first order arteries where the responses to both NA and α,β-methyleneATP were enhanced in the presence of U46619. Further investigations under raised tone conditions showed that the nerve-mediated responses were inhibited by either prazosin or α,β-methyleneATP showing the involvement of NA and ATP in the mediation of the neurogenic response.

In chapter 4 I demonstrated that L-type $Ca^{2+}$ cannels were involved in the mediation of the responses α,β-methyleneATP under raised tone conditions. Therefore, in the present study I examined a possible involvement of L-type $Ca^{2+}$ cannels in the mediation of nerve-mediated responses in porcine

mesenteric small arteries. Nifedipine inhibited the enhanced responses in the presence of U46619 indicating that L-type $Ca^{2+}$ channels may have role in the mediation of the enhanced responses in porcine mesenteric small arteries.

However, other studies have shown no role for L-type $Ca^{2+}$ channels in response to neuronally evoked purinergic response in rat small mesenteric arteries, and it was suggested that enough calcium enters the cell through P2X receptors to cause contraction (Gitterman and Evans, 2001). Similar results were also observed for exogenously applied α,β-methyleneATP, for example, in submucosal arterioles of guinea pig ileum (Galligan et al., 1995). It should be noted that these experiments were conducted under basal tone conditions; indeed they are consisting with the results obtained in chapter 4 where it has been shown that L-type $Ca^{2+}$ cannels were not involved in the mediation of the response to α,β-methyleneATP since nifedipine failed to inhibit the responses under basal tone conditions. These results demonstrate that the role of P2X receptors and voltage-sensitive calcium channels can be underestimated in the absence of appropriate tone conditions which may explain the absence of the purinergic component of the nerve-mediated responses obtained in this present study under basal tone conditions.

One of the aims of the present study was to compare the size of the purinergic component in porcine mesenteric first order and small sized arteries. Data obtained in the present study clearly demonstrated that the purinergic response increases as the size of the arteries decreases tone. This is in line with the demonstration that in rat large (first order) mesenteric arteries P2X receptors were about 100 times less sensitive to agonist (α,β-methyleneATP) than in

smaller arteries , attributed to an increased number of P2X receptors in the small arteries (Gitterman and Evans, 2000). Furthermore, this is consistent with results obtained in rat mesenteric arteries where the purinergic component of EFS was larger as the size of the arteries decreases (Gitterman and Evans, 2001). On the other hand, smaller purinergic response in large arteries should not be a surprise, as it has been demonstrated using [$^3$H] α,β-methyleneATP radioligand, that in rat, guinea pig and human pulmonary arteries the density of P2X receptors was higher in medium- and small-sized arteries than in that of large arteries (Zhao et al., 1996).

In conclusion, data obtained in the current study showed that under conditions of raised tone the nerve-mediated response was enhanced through the enhancement of purinergic and noradrenergic component via a postjunctional mechanism. This enhancement was in association with L-type $Ca^{2+}$ channels. The purinergic response of porcine mesenteric arteries is more evident as the size of the arteries becomes smaller.

# CHAPTER 6

# PRE-CONSTRICTION INCREASES NERVE-MEDIATED RESPONSES IN RAT PRESSURIZED MESENTERIC ARTERIES

## 6.1 INTRODUCTION

In vivo, blood vessels constrict on the background of variable degrees of intraluminal pressure. It has been demonstrated that exposing blood vessels to different levels of blood pressure alters vascular responsiveness. For example, increasing pressure from 20 mmHg to 60 mmHg enhanced the sensitivity of responses to noradrenaline in rabbit mesenteric arteries (Dunn et al., 1994). With regard to nerve-mediated responses, increasing the intraluminal pressure from 30 mmHg to 90 mmHg in rat mesenteric arteries made responses larger and revealed a predominant functional role for adenosine triphosphate (ATP) as the sympathetic neurotransmitter in rat mesenteric arteries (Rummery et al., 2007). In chapter 3, 4 and 5 I demonstrated that raising tone with U46619, a thromboxane mimetic agent, enhanced the responses to EFS and exogenous NA and α,β-methyleneATP in porcine mesenteric arterial bed and different sized porcine mesenteric arteries. Thus it seems that both pressure and tone play an important role in the sympathetic control of blood vessels. Therefore, the aim of the present study was to investigate the responses to nerve stimulation and the contribution of NA and ATP in rat or porcine small mesenteric arteries under the combined effects of pressure with or without pre-constriction using U46619.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Rat mesenteric arteries preparation

Male Wistar rats (200–250 g) were stunned by a blow to the cranium and killed by exsanguination. Second order mesenteric arteries were set up for pressure myograph recording as described in section 2.5.

### 6.2.2 Porcine mesenteric arteries preparation

Porcine mesenteries were obtained as described in section 2.1. Fine dissection was carried out to identify the small terminal branch arteries using a dissecting microscope. The porcine arteries were then set up as described in section 2.6.

### 6.2.3 Responses to EFS in rat pressurized mesenteric arteries under basal tone conditions

Following a 20 min equilibration period, arteries were stimulated at 10 Hz (50 pulses) at 5 min intervals and the stimulation voltage was progressively increased until the maximal response was produced, usually between 10-20 V. Frequency response curves (FRCs) to 50 pulses at frequencies of 0.5, 2 and 10 Hz were then constructed. Following each train of stimuli, the diameter of the artery was allowed to return to the baseline value before the next stimulus was applied. Two FRCs were obtained, at intervals of 30 min, in the absence of drugs to act as time controls. In some experiments, after the first FRC (control), YM-12617 (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, or NF 449 (0.1

μM), a $P2X_1$ receptor antagonist, were added to examine their effect on electrically-evoked vasoconstrictor responses. These concentrations of antagonists were chosen since they have previously been shown to selectively antagonise $\alpha_1$-adrenoceptor and P2X-receptor-mediated functional responses in mesenteric arteries (Fujii and Kuriyama, 1985, Braun et al., 2001, Kassack et al., 2004). YM-12617 was used instead of prazosin because preliminary experiments showed that prazosin sticks to the tubing of the pressure myograph system making it difficult to wash it out.

### 6.2.4 Responses to EFS in rat pressurized mesenteric arteries under raised tone conditions

The thromboxane $A_2$ agonist U46619 (2-10 nM) was used to reduce the diameter of pressurized arteries held at 90 mmHg, by about 15 – 20% of their initial diameter. In the presence of U46619, two FRCs were constructed to investigate the reproducibility of the electrically-evoked vasoconstrictor responses. In some experiments, after the first FRC (control), YM-12617 or NF 449 were added to test their effect on electrically-evoked vasoconstrictor responses. Both YM-12617 and NF 449 were perfused for 30 min before constructing the second FRC.

### 6.2.5 Responses to exogenous noradrenaline and α,β-methyleneATP in rat pressurized mesenteric arteries under basal and raised tone conditions

A concentration response curve (CRC) to exogenous NA was constructed under basal tone conditions, in rat mesenteric arteries held at 90 mmHg. The

tissue was then washed with physiological salt solution for 30 min, during which the perfusate was circulated to waste. U46619 was then added to pre-constrict the vessels by about 15 – 20% of their initial diameter. When a stable contraction to U46619 was achieved a second CRC to NA was constructed.

In separate experiments a single concentration of α,β-methyleneATP (0.1 μM) was added under basal tone conditions in rat mesenteric arteries held at 90 mmHg. The tissue was then washed with physiological salt solution for 30 min during which the perfusate was circulated to waste. U46619 was then added to pre-constrict the vessel by about 15% – 20% of their initial diameter. When a stable contraction to U46619 was achieved a second single application of α,β-methyleneATP (0.1 μM) was applied. Similar experiments were carried out in porcine small mesenteric arteries. Unfortunately, no response to EFS was observed in these vessels, although they did respond to α,β-methyleneATP.

## 6.3 STATISTICAL ANALYSIS

Results are expressed as the mean ± S.E.M. Statistical comparisons were made by two way analysis of variance (ANOVA) with Bonferroni post-hoc test or Student's paired t-test if the data were normally distributed (checked by Shapiro-Wilk normality test) while Mann-Whitney test was used if the data were not normally distributed. A value of $P < 0.05$ was taken to indicate statistical significance.

## 6.4 RESULTS

### 6.4.1 Effects of $\alpha_1$-adrenoceptor and $P2X_1$ receptor antagonists on vasoconstrictor responses to EFS in rat pressurized mesenteric arteries under basal tone conditions

The inner diameter of the second-order mesenteric arteries pressurized to 90 mmHg was 331 ± 8 μm (n =33). There was no spontaneous decrease in vessel diameter during the equilibration period. EFS of the perivascular nerves produced vasoconstrictor responses that increased in amplitude with an increase in stimulation frequency. Under basal tone conditions, two FRCs were reproducible (n=7) (Fig. 6.1). YM-12617 (0.1 μM), an $\alpha_1$-adrenoceptor antagonist, almost abolished the response at 0.5 and 2 Hz and significantly inhibited the electrically-evoked vasoconstrictor responses at 10 Hz (by 83 ± 6%, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=4) (Fig. 6.2A). NF 449 (0.1 μM), a $P2X_1$ receptor antagonist, was less effective but substantially inhibited the electrically-evoked vasoconstrictor responses at 10 Hz (by 70 ± 8%, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=7) (Fig. 6.2B).

### 6.4.2 Effects of $\alpha_1$-adrenoceptor and $P2X_1$ receptor antagonists on vasoconstrictor responses to EFS in rat pressurized mesenteric arteries under raised tone conditions

U46619 (2-10 nM) reduced the internal diameter of the rat pressurized mesenteric arteries (by 21 ± 5%) and enhanced responses to EFS at all frequencies (e.g. by 100 ± 19% at 10 Hz, $P < 0.001$, ANOVA followed by

Bonferroni post-hoc test) (n=14) (Fig. 6.3A and B). Under raised tone conditions two FRCs were reproducible (n=10) (Fig. 6.4). YM-12617 (0.1 μM) inhibited the vasoconstrictor responses to EFS (by 65 ± 9% at 10 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=8) (Fig. 6.5A). However, there was a larger YM-12617-resistant component than that evident under basal tone conditions. NF 449 (0.1 μM) inhibited the electrically-evoked vasoconstrictor responses (by 53 ± 10% at 10 Hz, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 6.5B) to a similar extent to that seen under basal conditions (Fig. 6.2B).

### 6.4.3 Responses to exogenous NA and α,β-methyleneATP in rat pressurized mesenteric arteries under basal and raised tone conditions

Exogenous NA produced vasoconstrictor responses which were concentration-dependent. After inducing tone with U46619 these responses were enhanced (e.g. from 50 ± 17 μm to 174 ± 25 μm at 1 μM of NA, $P < 0.001$, ANOVA followed by Bonferroni post-hoc test) (n=5) (Fig. 6.6A and B). Similarly, responses to α,β-methyleneATP (0.1 μM) were increased ($P < 0.01$, Student's paired t-test) (n=7) (Fig. 6.7A and B) in the presence of U46619.

### 6.4.4 Effects of α,β-methyleneATP in the porcine small mesenteric arteries pressurized at 90 mmHg under basal and raised tone conditions

Although I demonstrated in chapter 5 that porcine small mesenteric arteries were rich in sympathetic perivascular nerves (see Fig. 5.2A) and they

responded to EFS under isometric conditions, I failed to evoke vasoconstrictor responses to EFS in the pressurised small porcine mesenteric arteries. However, U46619 contracted the porcine small mesenteric arteries by 109 ± 49 μm. Furthermore, α,β-methyleneATP (0.1 μM) produced a contraction in these vessels that was increased although statistically it was not significant ($P > 0.05$ Mann Whitney test) (n=4) (Fig. 6.8) in the presence of U46619-induced tone.

There is no clear reason to why the porcine pressurised arteries did not respond to EFS. It may be that the lack of fat and connective tissues that covers the vessel to enable a tight seal in the suction electrode was insufficient. Nevertheless, in some experiments small responses to EFS could be generated but only at a very high voltage and with high frequencies and these were not sensitive to guanethidine.

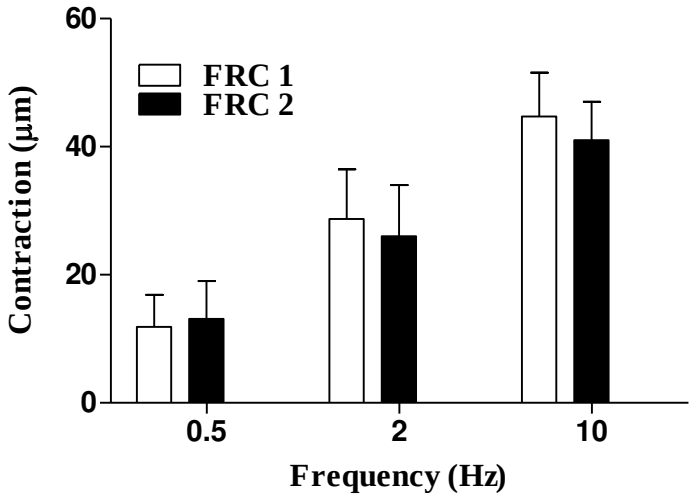


Fig. 6.1 Reproducibility of vasoconstrictor responses to electrical field stimulation (0.5-10 Hz, 50 pulses, 10-20 V) at 90 mmHg. Opened bars show the first frequency response curve (FRC 1) while closed bars show second frequency response curve (FRC 2) (n=7) in rat pressurized mesenteric arteries under basal tone conditions. Each bar represents mean ± standard error.

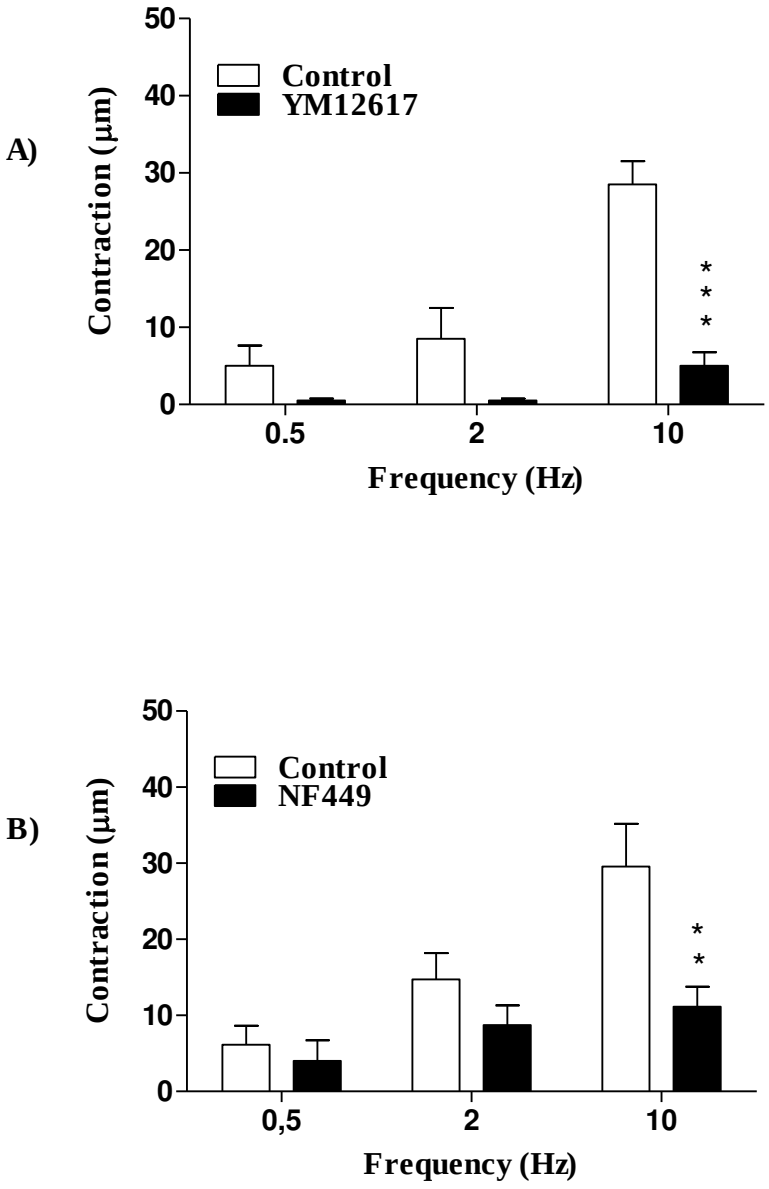


Fig. 6.2 Effects of A) YM-12617 (0.1 µM) (n=4), or B) NF499 (0.1 µM) (n=7), on responses to electrical field stimulation (0.5-10 Hz, 50 pulses, 10-20 V) mmHg in rat mesenteric arteries pressurized to 90 mmHg under basal tone conditions. Each bar represents mean ± standard error. ** $P < 0.01$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

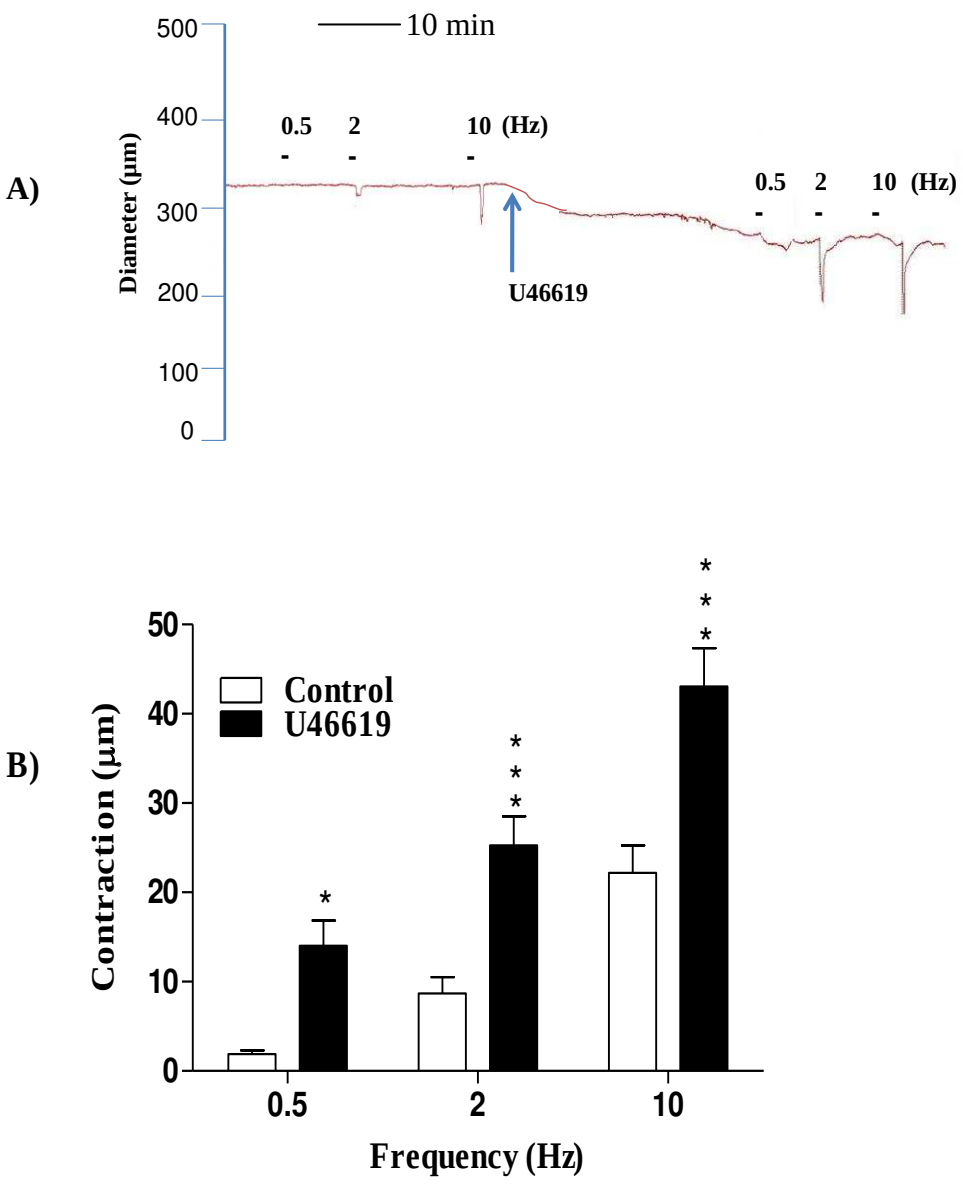


Fig. 6.3 A) Representative trace showing neuronally evoked contractile responses under basal tone conditions and in the presence of U46619 in rat mesenteric arteries pressurised at 90mmHg. B) Summary data of contractile responses to electrical field stimulation (0.5-10 Hz, 50 pulses, 10-20 V) under basal tone conditions (control), and in the presence of U46619 (2-10 nM) (n=14) in rat mesenteric arteries pressurized to 90 mmHg. Each bar represents mean ± standard error.* $P < 0.05$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

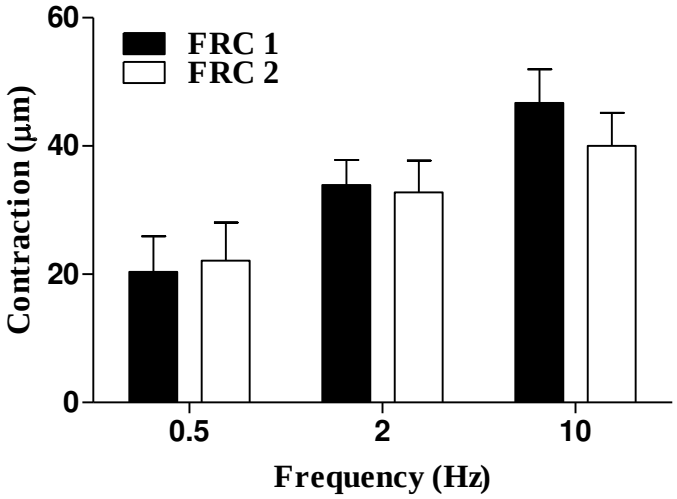


Fig. 6.4 Reproducibility of vasoconstrictor responses to electrical field stimulation (0.5-10 Hz, 50 pulses, 10-20 V) at 90 mmHg. Opened bars show the first frequency response curve (FRC 1) while closed bars show second frequency response curve (FRC 2) (n=10) in rat pressurized mesenteric arteries under raised tone conditions. Each bar represents mean ± standard error.

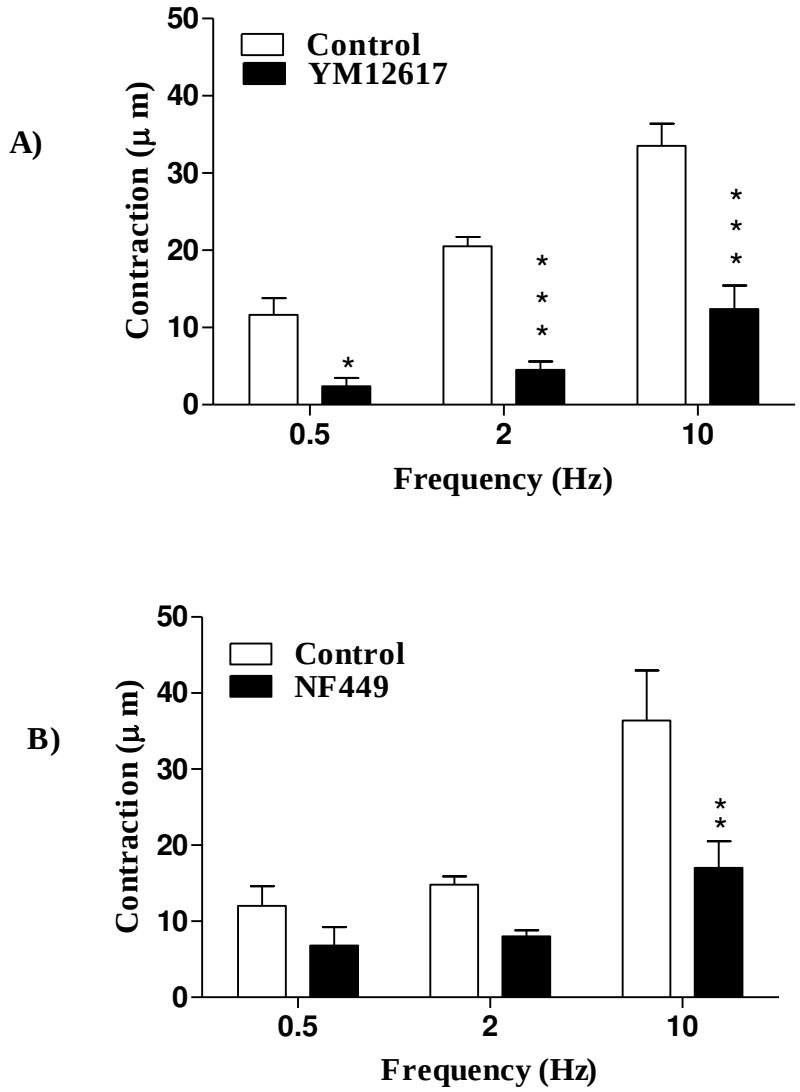


Fig. 6.5 Effects of A) YM12617 (0.1 μM) (n=8), or NF449 (0.1 μM) (n=5) on responses to electrical field stimulation (0.5-10 Hz, 50 pulses, 10-20 V) in rat mesenteric arteries pressurized to 90 mmHg under raised tone conditions. Each bar represents mean ± standard error.* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

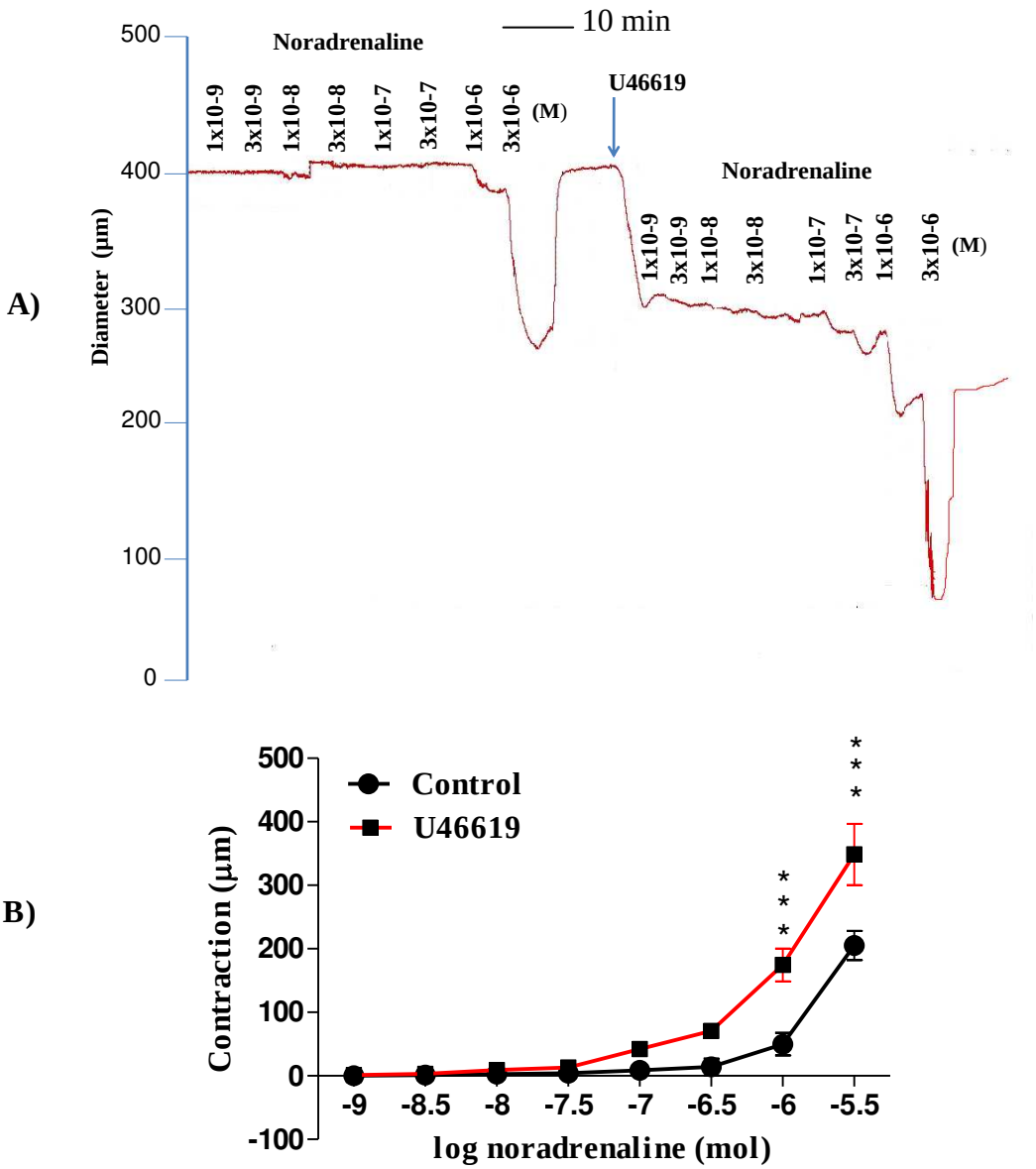


Fig. 6.6 A) Representative trace showing application of exogenous NA (cumulative response curve) in the absence (basal tone conditions) and in the presence of U46619 (raised tone conditions) in rat mesenteric arteries pressurised to 90mmHg. B) Summary data of responses to NA (n=5), under basal tone conditions (control), and in the presence of U46619 (4-10 nM) in rat mesenteric arteries pressurized to 90 mmHg. Each bar represents mean ± standard error. *** $P < 0.001$ vs. control (ANOVA followed by Bonferroni post-hoc test).

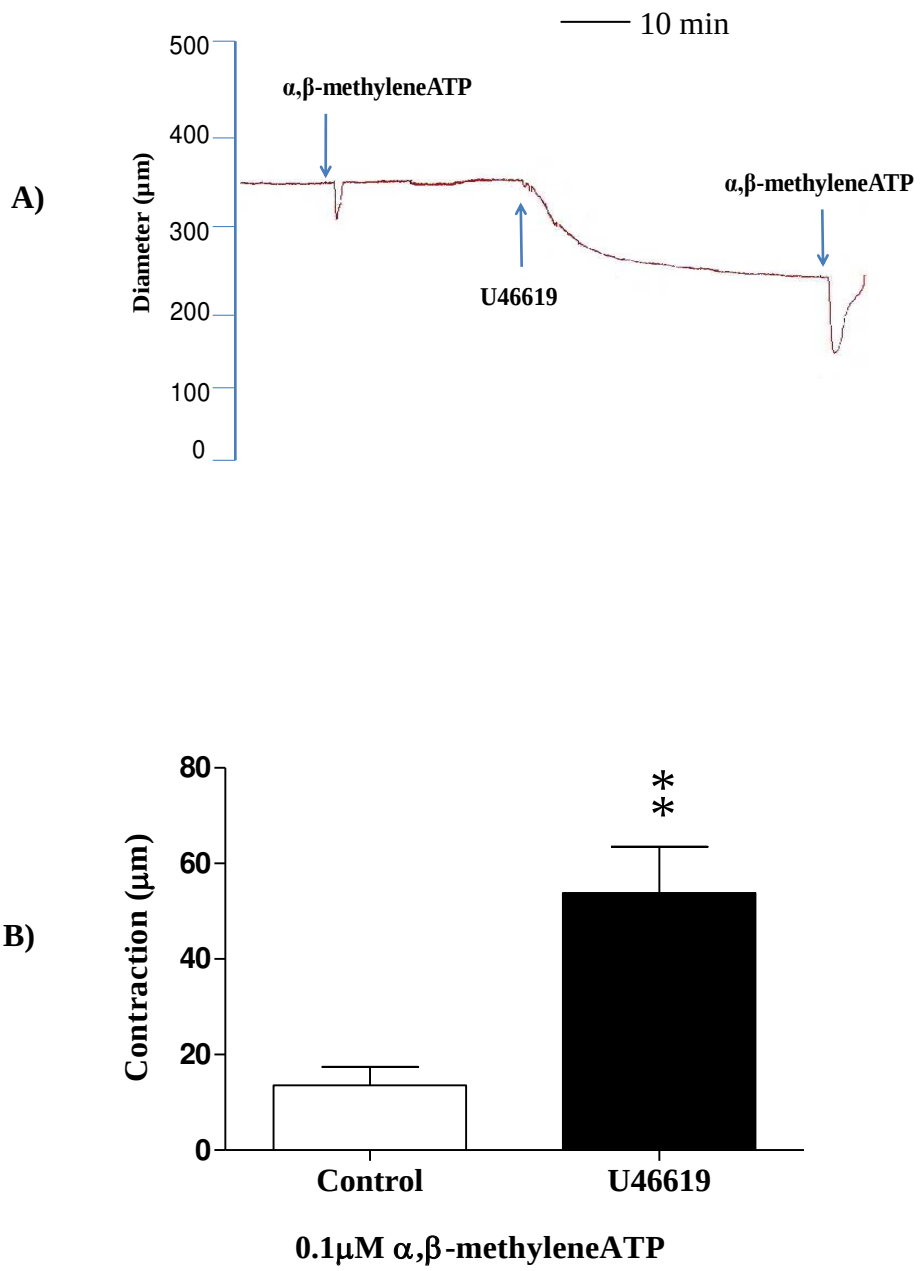


Fig. 6.7 A) Representative trace showing a single application of α,β-methyleneATP (0.1 µM), and in the absence (basal tone conditions) and in the presence of U46619 (raised tone conditions), in rat mesenteric arteries pressurised at 90 mmHg. B) Summary data of responses to α,β-methyleneATP (0.1 µM) (n=7) under basal tone conditions (control), and in the presence of U46619 (2-10 nM) in rat pressurized mesenteric arteries at 90 mmHg. Each bar represents mean ± standard error. ** P < 0.01 vs. control (Student's paired t test).

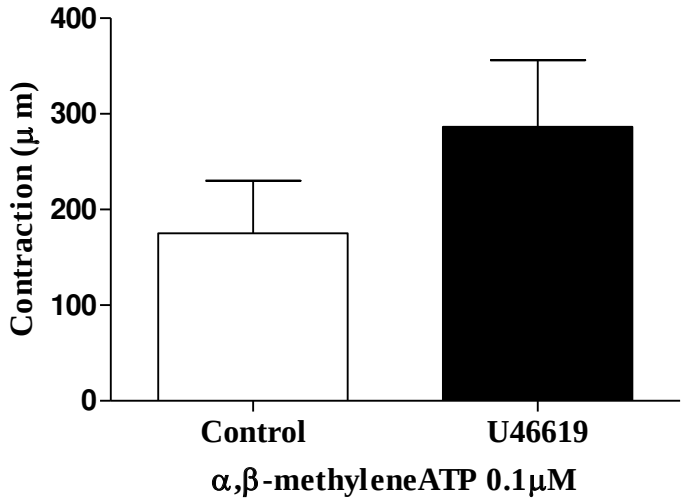


Fig. 6.8 Effects of α,β-methyleneATP (0.1 µM) (n=4), under basal tone conditions (control), and in the presence of U46619 (2-10 nM) in porcine small mesenteric arteries pressurized to 90 mmHg. Each bar represents mean ± standard error. P > 0.05 (Mann Whitney test).

## 6.5 DISCUSSION

The main finding of the present study is that in rat mesenteric arteries pressurised at 90 mmHg and pre-constricted with U46619, responses to electrically-evoked and exogenously applied NA and α,β-methyleneATP were enhanced. Furthermore, both NA and ATP contributed in the mediation of the electrically-evoked vasoconstriction through activation of $\alpha_1$-adrenoceptors and $P2X_1$ receptors respectively, under basal, and raised tone conditions.

The aim of the present study was to investigate the sympathetic neurotransmission of isobarically mounted porcine mesenteric arteries using EFS. Unfortunately, this was not possible although application of α,β-methyleneATP evoked vasoconstrictor responses indicating that the vascular preparations were viable and contained P2X receptors. Furthermore, I demonstrated in chapter 5 that porcine mesenteric small arteries are rich in sympathetic perivascular nerves and responded to nerve activation when mounted on isometric myograph. In addition, it is unlikely that the lack of a response was due to technical limitations, since responses were obtained in similar sized rat mesenteric arteries (see below). Thus, it is surprising no responses were obtained to EFS in porcine mesenteric small arteries. It is possible that the difficulty in getting these vessels to form a tight seal in the suction electrode, because of the lack of fat and connective tissues that cover the vessels, may have impacted.

Nevertheless, α,β-methyleneATP evoked vasoconstrictor responses in porcine small mesenteric arteries under basal tone conditions. These vasoconstrictor

responses were enhanced in the presence of U46619. This is consistent with the results obtained in isometrically mounted porcine mesenteric small arteries (see chapter 5).

In the absence of sustainable response to EFS in porcine tissue, I examined the effects of applying tone with U46619 in rat mesenteric arteries held under pressure since both mechanisms have been shown to enhance purinergic signalling in response to EFS (chapters 3, 4 and 5 and see Rummery et al). Under pressurised conditions (no tone), vasoconstrictor responses were sensitive to both YM-12617, a selective $\alpha_1$-adrenoceptor antagonist, and NF 449, a selective $P2X_1$ receptor antagonist. This indicates that both NA acting through postjunctional $\alpha_1$-adrenoceptors and ATP acting via $P2X_1$ receptors are involved in mediating these responses.

Under pressurised conditions (in the presence of tone), U46619 significantly enhanced the electrically-evoked vasoconstrictor responses. This has also been reported in human saphenous vein where U46619 enhanced responses to electrically-evoked vasoconstrictor responses (Vila et al., 2001), and is consistent with the results obtained in the porcine mesenteric small arteries (see chapter 5). Under these conditions, YM-12617 inhibited the electrically-evoked vasoconstrictor responses indicating that NA is involved in the mediation of these responses through postjunctional $\alpha_1$-adrenoceptors. Furthermore, in the presence of U46619, NF 449 inhibited the electrically-evoked vasoconstrictor responses indicating that ATP is involved in the mediation of the responses through $P2X_1$ receptors. It should be noted that there was a slightly larger YM-12617-risistant component (purinergic

component) and NF 449-risistant component (noradrenergic component) in the presence of U46619 than that obtained under basal tone conditions. This may suggest that U46619 enhanced the YM-12617-resistant component (purinergic component) and NF 449-resistant component (noradrenergic component) which is consistent with the enhanced responses to the exogenous applications of NA and α,β-methyleneATP in the presence of U46619 obtained in the present study.

The mechanism by which U46619 enhanced electrically-evoked vasoconstrictor responses seems to be through a postjunctional mechanism, since U46619 enhanced responses to exogenously applied NA in the rat pressurised mesenteric arteries at 90 mmHg. This is consistent with the results of other experiments where U46619 enhanced responses to exogenously applied NA in human saphenous vein (Vila et al., 2001), and human umbilical vein (Errasti et al., 2007) and is consistent with the results obtained in the porcine mesenteric small arteries (see chapter 5). U46619 also enhanced responses to α,β-methyleneATP which is consistent with results obtained in the rat perfused arterial bed where responses to α,β-methyleneATP were larger in pre-constricted preparations (Pakdeechote et al., 2007) and parallels my observation in porcine perfused arterial bed (see chapter 3).

Although YM-12617 inhibited the response more than NF 449 especially at lower frequencies, both antagonists decreased responses by greater than 50% especially at higher frequencies under basal and raised tone conditions, indicating that NA and ATP may be acting synergistically. Synergism between NA and ATP has been reported in rat mesenteric arteries (Ralevic and

Burnstock, 1990). The mechanisms of synergism are not well understood. Nevertheless, it has been suggested that in the vas deferens smooth muscle NA potentiating the contractile responses to ATP by sensitising smooth muscle cells to calcium through the inhibition of myosin light chain phosphatase (Burnstock, 2004).

Other studies of the native $P2X_1$ receptor have used NF-449 at concentrations ranging from 1 to 10 μM (Kassack et al., 2004). Nevertheless the concentration of drug used in the present study caused a greater than 50% inhibition, thus it is unlikely a higher concentration would have altered the nature of the results obtained (i.e. a synergistic interaction between NA and ATP).

In conclusion it seems that the combination of appropriate pressure and tone leads to an enhanced sympathetic response that is not mediated by a particular sympathetic neurotransmitter but by the involvement of both NA and ATP. ATP contributed to nerve-mediated vasoconstrictor responses in a synergistic way. U46619 enhances nerve-mediated responses and provides a synergistic stimulus for NA and ATP possibly through a postjunctional mechanism.

# CHAPTER 7

# GENERAL DISCUSSION

An important role for ATP as a sympathetic neurotransmitter is known. However, the relative importance of ATP as a functional sympathetic neurotransmitter in blood vessels has been shown to be variable. This variability occurs due to a number of factors including species, vascular bed, size of blood vessel and the level of pre-existing vascular tone (Ralevic, 2009). Vascular tone reflects the degree of constriction the blood vessels experiences relative to its maximally dilated state under basal tone conditions. Nevertheless, in most studies consideration of vascular tone is absent. Recent studies showed that in the rat perfused mesenteric bed in which vascular tone was raised using U46619, ATP became an important functional sympathetic neurotransmitter (Pakdeechote et al., 2007). The current study investigated if raised vascular tone would reveal a functional role for ATP as a sympathetic neurotransmitter in porcine perfused mesenteric bed and isolated mesenteric arteries, and demonstrated this to be the case.

One of the important observations in this study was that enhanced contractile responses to EFS were obtained under conditions of raised tone induced by U46619. This enhancement was evident in the porcine perfused vascular bed and in isometrically mounted porcine mesenteric arteries of different sizes. To further investigate the nature of the electrically-evoked contractile responses I examined the responses during $\alpha_1$-adrenocetor and/or P2X receptor blockade and showed that they were mediated solely by NA via the activation of postjunctional $\alpha_1$-adrenocetors under conditions of basal tone. Similar results have been reported in the isolated perfused mesentery of the rat (Williams and

Clarke, 1995, Pakdeechote et al., 2007). Conversely, raising tone with U46619 revealed that these responses were partially mediated by ATP through the activation of P2X receptors. This observation was evident in the perfused vascular bed and in isolated arteries of different sizes. Thus, raising tone with U46619 enhanced the purinergic response and as a consequence the whole response was enhanced.

Further investigations showed that the purinergic response was enhanced under conditions of raised tone via a postjunctional mechanism since responses to the analogue of ATP, α,β-methyleneATP, were enhanced under conditions of raised tone in the perfused bed and isometrically mounted arteries of different sizes. Furthermore, the enhancement of the purinergic response seems to be a consequence of raising the tone regardless of the agent used to induce the tone, since enhanced responses to α,β-methyleneATP were also obtained in the presence of ET-1. It has been shown that U46619 depolarizes vascular smooth muscle (Crane and Garland, 2004, Shaw et al., 2004). Thus, any depolarization caused by U46619 may provide the conditions where further depolarization caused by ATP acting on P2X receptors results in a more substantial opening of $Ca^{2+}$ channels and a contraction (Pakdeechote et al., 2007). Some data obtained in the present study supports this suggestion since nifedipine attenuated responses evoked by EFS and α,β-methyleneATP under raised tone condition but not under basal tone conditions.

An enhancement of the electrically-evoked contractile responses through the enhancement of the noradrenergic component of the sympathetic response under conditions of raised tone may also occur although this was hard to define

for several reasons. Firstly, NA was the major neurotransmitter under basal and raised tone conditions, thus making it difficult to define an enhanced response although responses to exogenous NA were potentiated with U46619. Secondly, electrically-evoked NA may act not only on $\alpha_1$-adrenoceptors but also on postjunctional $\alpha_2$-adrenoceptors and prejunctional $\alpha_2$-adrenoceptors which are known to inhibit the release of NA may cause underestimation of postjunctional inhibition.

Another mechanism by which U46619 may enhance the contractile responses is by increasing the myofilament $Ca^{2+}$ sensitivity in vascular smooth muscles (Ungvari and Koller, 2000, Ding and Murray, 2005). However, data obtained in the current study showed that nifedipine inhibited the enhanced electrically-evoked contractile responses and the enhanced responses to exogenous α,β-methyleneATP. Thus the enhanced responses required entry of extracellular $Ca^{2+}$ through the L-type calcium channels. Moreover, α,β-methyleneATP inhibited the enhanced responses indicating that extracellular calcium entry via P2X receptors was required. Thus our data show that the enhanced responses appeared to utilize calcium mainly extracellular rather than from intrarcellular stores. However, there is a possibility that calcium entering via P2X receptors or/and calcium-sensitive channels may activate the calcium-induced calcium release mechanism which clearly needs further investigations.

Although data obtained in this study indicate a postjunctional mechanism of enhancement of the electrically-evoked contractile responses a prejunctional mechanism cannot be excluded. The use of new methods such as the $Ca^{2+}$

confocal imaging (Brain, 2009, Wier et al., 2009) would be useful in further investigations of the release of NA and ATP in future studies.

It is well known that smaller arteries are more involved in the control of blood pressure than large conduct arteries. It has been reported that the density of P2X receptors is larger as the size of blood vessels decreases in the rat mesenteric vascular bed (Gitterman and Evans, 2000). Furthermore, an increasing role for P2X receptors in mediating electrically-evoked contractile responses in rat mesenteric arteries as the size of blood vessels decreases has also been shown (Gitterman and Evans, 2001). In this study I demonstrated that responses to α,β-methyleneATP were more prominent in porcine small arteries than those in larger arteries. Moreover, the purinergic component of EFS seems to be larger in porcine small arteries than in larger arteries. However, the purinergic component of the contractile responses to EFS was evident only under the conditions of raised tone, and seemed modest if compared to that obtained in the study of Gitterman and Evans (2001) in rat mesenteric arteries. Thus, data obtained in this study seems to be in agreement with a growing consensus that the purinergic component of the sympathetic neurotransmission increases as the size of vessel decreases. The revelation that ATP has a prominent functional role in these arteries highlights the potential importance of ATP in the regulation of blood pressure.

It has been reported that isobarically mounted rabbit mesenteric arteries were more sensitive to physiological concentrations of agonists than when they were isometrically mounted (Dunn et al., 1994). In addition, increasing the intraluminal pressure from 30 mmHg to 90 mmHg in rat mesenteric arteries

made responses larger and revealed a predominant functional role for ATP as the sympathetic neurotransmitter in rat mesenteric arteries (Rummery et al., 2007). Furthermore, data obtained in this study showed that contractile responses in vessels exposed to pressure in porcine perfused mesenteric bed are enhanced more than those in isometrically mounted arteries. It has also been previously reported that increasing pressure depolarizes vascular smooth muscle and increases the amplitude of the excitatory junctional potential (Rummery et al., 2007). Raising vascular tone enhanced the electrically-evoked contractile responses of the rat perfused mesenteric bed (Pakdeechote et al., 2007) and porcine mesentery (this thesis). Thus, I investigated the effects of exposing rat small mesenteric arteries to the combined effects of pressure (close to that experienced in vivo which is approximately between 80 and 115 mmHg (Rummery et al., 2007)) and a degree of pre-constriction (tone), and showed an enhancement of the electrically-evoked vasocontractile responses.

Under basal tone conditions, further investigations in rat pressurised mesenteric arteries showed that the electrically-evoked vasocontractile responses were sensitive to the selective $\alpha_1$-adrenoceptor antagonist, YM-12617, as well as to the selective $P2X_1$ receptor antagonist, NF 449. This indicates that both NA acting through postjunctional $\alpha_1$-adrenoceptor and ATP acting via $P2X_1$ receptors are involved in mediating these responses.

Under conditions of raised tone with U46619, the electrically-evoked vasocontractile responses were enhanced. However, further investigations showed that enhanced vasocontractile responses were not mediated by a particular sympathetic neurotransmitter but by the involvement of both NA and

ATP. Furthermore responses to exogenous NA and α,β-methyleneATP were also enhanced in pressurised arteries under raised tone conditions suggesting a postjunctional mechanism of enhancement. These results clearly demonstrated an important role for ATP as a sympathetic neurotransmitter provided similar conditions of tone and pressure to those experienced in vivo are considered. The association of increased blood pressure and role for ATP as a sympathetic neurotransmitter has also been demonstrated in animal models of hypertension. For example, in tail arteries of spontaneous hypertensive rat (SHR) a dominance of the purinergic component of the sympathetic response has been reported (Vidal et al., 1986). Furthermore, an increased responsiveness to α,β-methyleneATP in the blood vessels of SHR perfused kidneys has been shown (Fernandez et al., 2000). In addition, contractile responses to ATP were enhanced in SHR aorta (Yang et al., 2004). Thus, it seems that data obtained in this study are in agreement with the growing evidence on the involvement of ATP as a functional sympathetic neurotransmitter both in animal models of hypertension and in experiments where the conditions of pressure and tone are similar to that experienced in vivo.

In summary, this study demonstrated that under conditions of basal tone NA was the main neurotransmitter mediating the electrically-evoked contractile responses in the porcine perfused whole mesenteric and in porcine mesenteric arteries of different sizes. Conversely, when tone was raised ATP contributed as a functional sympathetic neurotransmitter in the mediation of the responses. This contribution seems to be more evident as the size of the blood vessel decreases. In rat mesenteric arteries pressurised close to physiological levels

and exposed to pre-constriction ATP and NA both contributed in the mediation of the electrically-evoked contractile responses. These results could have important implications for our understanding of the sympathetic control of blood vessels and elevated blood pressure that occurs in hypertension.

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